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Physiological Zoölogy

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ORGANIC MATTER IN DISSOLVED AND IN COLLOIDAL FORM AS FOOD FOR DAPHNIA MAGNA[1]

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I

THE organic matter present in lake water and in sea water exists in greater quantity as dissolved material than as components of the bodies of plankton organisms (Birge and Juday, 1926, 1927; Bond, 1933). The presence of amino acids makes it probable that the nitrogen compounds contained in the dissolved material are suitable for animal nutrition (Peterson, Fred, and Domogalla, 1925). The possibility therefore exists that aquatic organisms may utilize the dissolved material in the water for food, but the question of whether this is actually the case can be decided by direct experiment only.

Certain organisms have been shown to absorb organic substances from fairly concentrated solution (e.g. tadpoles and mussels; Kříženecký, 1925) but in the words of Krogh (1931) "there is no convincing evidence that any animal takes up dissolved organic substances from *natural* waters in any significant amount. . . . . It is possible according to the experiments of Krogh that higher animals take up insignificant quantities of dissolved combustible material, but the quantities excreted are always larger." However, certain in-

[1] The authors wish to acknowledge their indebtedness to Professor W. J. Crozier for helpful criticism, to Professor W. H. Weston for the use of his culture room, and to Dr. A. B. Keys for the loan of the ultra-filter.

vestigators—notably Pütter (1907, 1909)—have maintained the contrary. But many of the earlier experiments were invalidated by failure to recognize the possibility that bacteria were acting as a source of food, as has been shown by Bond (1933). The present experiments were designed to test Pütter's theory for *Daphnia magna*, a convenient laboratory plankton animal, special precautions being taken against the influence of bacteria. Furthermore, an aspect of the problem is presented which has not hitherto received sufficient emphasis, namely, the utilization for food of the organic matter which exists in the water in colloidal form.

The first experiments on the utilization of dissolved organic matter by Cladocera were carried out by Knörrich (1901). *Daphnia* were placed in a sterilized and filtered hay infusion which was changed daily. The animals lived about two weeks and released young which in turn grew in the medium. Wolff (1909) showed, however, that centrifuging such a "solution" revealed a mass of fine particles with which he was able to feed *Daphnia*. But at the same time Wolff found that the adult females of *Simodaphnia*, when placed in filtered aquarium water, released young which in turn developed. Since neither the solutions nor the animals were sterile, the possibility that bacteria were being used as food was not excluded. Kerb (1910) repeated Wolff's experiments, taking greater care to prevent the growth of bacteria, and found that the *Daphnia* molted readily but that their weight was reduced to a quarter of its former amount. On the basis of a very limited investigation Krogh (1930) reported that *Daphnia pulex* can take up glucose from artificial solutions but that they always lose more dissolved matter than they take in.

II

In the present investigation a supply of *Daphnia* was kept on hand by culturing large numbers on media made from infusions of lettuce, of manure and soil (Banta, 1921), or of Fleischmann's yeast (Bond, 1934). The animals used in the experiments were sterilized by repeated washings in sterile tap water, using the same procedure as Stuart, McPherson, and Cooper (1931). The method of these investigators for testing the sterility of each flask at the conclusion of each experiment was employed. Each animal with a small amount

of the medium was transferred to a test tube containing double proteose-peptone broth and incubated at room temperature for two weeks. Any flask which was found not to have been sterile was eliminated from the results.

III

The nutritional requirements of *Daphnia* were studied first by investigating the effect of sterilizing and of removing the particulate

TABLE I

THE EFFECT OF STERILIZING AND OF FILTERING CULTURE MEDIA

Animal	Medium	No. of Tests	Percentage Releasing Young
SERIES I TAPWATER			
a) Sterilized	Unsterilized	10	20
b) Sterilized	Autoclaved (sterile)	10	0
c) Sterilized	Berkefeld filtered (sterile)	10	0
Control:			
Unsterilized	Unsterilized	17	23
Unsterilized	Autoclaved (sterile)	18	16
SERIES II POND WATER			
a) Sterilized	Unsterilized	14	71
b) Sterilized	Autoclaved (sterile)	12	25
c) Sterilized	Berkefeld filtered (sterile)	8	25
Control:			
Unsterilized	Unsterilized	19	73
SERIES III BANTA'S CULTURE MEDIUM			
a) Sterilized	Unsterilized	7	71
b) Sterilized	Autoclaved (sterile)	14	57
c) Sterilized	Berkefeld filtered (sterile)	9	44
Control:			
Unsterilized	Unsterilized	29	89
SERIES IV LETTUCE INFUSION			
a) Sterilized	Unsterilized	7	57
b) Sterilized	Autoclaved (sterile)	10	40
c) Sterilized	Berkefeld filtered (sterile)	9	33
Control:			
Unsterilized	Unsterilized	19	94

matter from the different culture media in which the animals ordinarily flourished. Individual sterilized *Daphnia* one day old were placed in cotton-stoppered Erlenmeyer flasks containing 150 cc. of media variously treated as indicated in Table I. All flasks were kept in an incubator at a temperature of 24° C.

The results show first that in all cases the percentage of animals

growing to maturity and releasing young in the autoclaved media is lower than that in unheated, non-sterile media. This may be due to the fact that *Daphnia magna* require living bacteria as food, or to the fact that protein material present in the water has been rendered unsuitable for food by heating. Second, in Series III and IV the percentage for Berkefeld-filtered media is lower than for autoclaved media. This shows that the presence of particulate food improves the condition of growth for *Daphnia*. However, the fact that 33–44 per cent of the animals did succeed in reaching maturity in the Berkefeld-filtered media proves conclusively that *Daphnia* can use filter-passing material as food. Stuart, McPherson, and Cooper (1931) reached the same conclusion for *Moina macrocopa*. Finally, it will be noted that for Berkefeld filtrates the larger percentages of animals releasing young occur in the cases of the media which are richer in filter-passing material. It is shown below that the filter-passing material consists both of colloids and of substances in solution.

Stuart, McPherson, and Cooper (1931) reported that the addition of sterile, particulate food (dead bacteria) enabled *Moina* to reach maturity in sterile tap water. We found that sterile *Daphnia* similarly could grow to maturity in filtered tap water if a small amount of autoclaved lettuce infusion (sterile) was added. Various known dissolved organic substances were then added to the sterile tap water in place of the particulate food. None of the *Daphnia* reached maturity, but the possibility still existed that dissolved material was actually being consumed but not to such an extent as to allow the animals to develop completely. The attainment of the second molt was therefore employed in a second series of tests as a criterion for growth, as was done by Bond (1933).

One-day-old *Daphnia* were sterilized as before and placed in the solutions of organic substances listed in Table II. Glucose was used because of all food substances it would seem the most easily assimilated. The remaining solutions are good nitrogen sources and it is such material which is most plentiful in lake water. The concentration of the glucose solutions was varied from a value equal to that found in lake water for dissolved organic matter to a value five times as great. The concentrations of the other substances tested were all

higher than would be expected in natural waters. In all cases the particular concentrations selected were those which seemed most favorable as judged by the length of life of *Daphnia* placed in them

TABLE II

SOLUTIONS OF ORGANIC SUBSTANCES AS FOOD FOR *Daphnia*

Sterility of Animals	Total No. of Tests	Medium	Percentage Attaining Second Molt
Sterilized	7	Berk. Filt. Glucose (st.)* conc: 13.3, 33.3, 66.6 mg./L.	0
Sterilized	8	Autocl. glucose solution (st.) conc: 66.6 mg./L.	0
Sterilized	9	Berk. Filt. glucose sol. (st.) conc: 33.3 mg./L.	0
Sterilized	22	Autocl. glucose sol. (st.) conc: 33.3 mg./L.	0
Sterilized	5	Berk. Filt. glucose sol. (st.) conc: 13.3 mg./L.	0
Sterilized	9	Autocl. glucose sol. (st.) conc: 13.3 mg./L.	0
Sterilized	8	Berk. filtered double proteose peptone broth (st.)	0
Sterilized	13	Autocl. double proteose peptone broth (st.)	0
Sterilized	7	Berk. Filt. peptone sol. (st.) conc: 33.3 mg./L.	0
Sterilized	9	Autocl. bactopeptone sol. (st.) conc: 33.3 mg./L.	0
Sterilized	9	Berk. filt. bactopeptone sol. (st.) conc: 66.6 mg./L.	0
Sterilized	22	Autocl. bactopeptone sol. (st.) conc: 66.6 mg./L.	0
Sterilized	5	Autocl. bactopeptone sol. (st.) conc: 100 mg./L.	0
Sterilized	7	Berk. filt. yeast autolysate sol. (st.) conc: 1–10	0
Sterilized	24	Autocl. yeast autolysate sol. (st.) conc: 1–10	0
Sterilized	3	Berk. filt. glucose sol. 66 mg./L. plus codliver oil conc: 1 per cent (st.)	0

* (st.)=sterile.

in preliminary experiments. In none of the solutions tested did the animals attain the second molt, thus showing that if *Daphnia* can use such types of dissolved organic matter at all, their value as a source of food must be a very minor one. Since practically 100 per cent of the animals reached the second molt in the experiments of

Table I, except in sterile tap water, we know that in the present case the *Daphnia* were not prevented from developing by the processes of sterilizing the organisms and the media.

IV

To ascertain whether *Daphnia* can utilize dissolved organic material in their active metabolism to some extent although they are not able to grow on it, solutions were tested for the removal of glucose after large numbers of *Daphnia* (of various ages, mostly females) had been present in them. Since sterilization of the necessary numbers of animals was impracticable, the influence of bacteria was prevented by causing the glucose solution to flow through the chamber in which the *Daphnia* were confined and measuring the concentration of the solution on entering and leaving the experimental chamber, using the Hagedorn-Jensen blood-sugar method. The same technique was employed by Krogh (1930), and similar arrangement of apparatus and rates of flow of the solution were used (200–2,000 cc. per hour). Sterile tap water was first passed through the apparatus for about 6 hours to remove as many of the bacteria as possible. The glucose solution was then allowed to flow, but no measurements were taken for 2 hours since glucose might be removed at first by simple absorption by the bodies of the animals.

The detailed observations of a typical experiment (Table III) show that a significant difference is the concentration of glucose between the ingoing and outgoing solutions did not occur at any time. With the method used absolute reliance can be placed only on differences of one division of the burette scale—representing 3.5 mg. glucose per liter—but measurements finer than this were estimated and have been entered in the table. It appears from the values obtained that a difference of 1.5 mg. per liter in the ingoing and outgoing solution would not have escaped detection. A possible, undetected difference between the inflow and the outflow of 1.5 mg. per liter would represent an uptake of 0.3 mg. of glucose by the *Daphnia* during the course of one hour at the slowest rate of flow (200 cc. per hour). The measurements of MacArthur and Baillie (1929) show that one gram of *Daphnia magna* (air-dried weight) produce an average of 1.89 mg. of CO_2 per hour. Assuming for the purposes of

a rough comparison that the animals were metabolizing glucose exclusively, 1.89 mg. CO_2 would represent the burning of 1.29 mg. of glucose. In our experiment there were 2.5 g. of *Daphnia* present. If these animals are comparable to those observed by MacArthur and Baillie, they would have required $2.5 \times 1.29 = 3.23$ mg. for their total metabolism during the course of one hour. Since we have calculated that the animals present did not consume more than 0.3 mg. of

TABLE III

MEASUREMENTS OF THE CONCENTRATION OF GLUCOSE IN THE INGOING AND OUTGOING SOLUTIONS OF A TYPICAL EXPERIMENT
(Air-dried weight of *Daphnia:* 2.5 grams.)

Test No.	Rate of Flow of Solution (Cc./Hr.)	Time from Start of Experiment (Min.)	Ingoing Glucose (Mg./L.)	Outgoing Glucose (Mg./L.)
1–1	200	0	20.1	20.4
1–2	200	15	20.5	20.5
1–3	200	30	19.8	20.2
1–4	200	45	20.0	20.0
1–5	300	60	20.0	20.0
1–6	300	75	20.0	20.0
1–7	400	90	19.4	19.7
1–8	400	105	20.6	20.1
1–9	400	120	20.0	20.0
1–10	400	135	20.2	20.3
1–11	400	150	20.4	20.4
1–12	500	165	20.6	20.4
1–13	500	180	19.3	20.2
1–14	500	195	20.1	19.7
1–15	500	210	20.2	20.2
1–16	500	225	21.1	21.4

glucose per hour we may conclude that the utilization of the glucose from the solution, if any, was certainly less in extent than 10 per cent of the animals' metabolism.

In eight other experiments, which are summarized in Table IV, and which comprise 160 observations, no significant differences were found in the concentration of glucose in the ingoing and outgoing solutions. However, Krogh (1931, p. 436) reported that certain animals, including *Daphnia pulex*, consumed glucose from solution to the extent of one-fifth of their metabolism. Since the supposed removal of glucose is no greater in amount than the variation

of the measurements, it does not appear justifiable to conclude from his data that any glucose has been taken up. Krogh himself says (1930, p. 163) "the experiments have come out variously and so should be repeated."

TABLE IV

SUMMARY OF EXPERIMENTS IN WHICH FLOWING SOLUTIONS OF GLUCOSE WERE USED

(In no case was a significant difference found in the concentration of glucose in the ingoing and the outgoing solutions.)

Exp.	Air-Dried Weight of Daphnia (Grams)	Glucose Conc. (Mg./L.)	Total No. of Tests	Rate of Flow of Solution (Cc./Hr.)
1	2.5	20	16	200–500
2	5.0	40	22	1,000–2,000
3	6.1	20	15	400–800
4	4.3	20	12	400–600
5	3.7	10	20	400–1,000
6	2.4	50	20	400–500
7	5.8	10	20	500
8	3.1	20	15	800
9	2.9	30	20	600–800

V

It has been shown above that *Daphnia* can develop to maturity in the Berkefeld filtrates of certain culture media. The filter-passing material consists both of substances in solution and of extremely fine matter in suspension, according to Hinman (1930) composed mainly of colloids. The experiments already described indicate that if dissolved organic matter can be used as food by *Daphnia* at all, its rôle is an insignificant one. Hinman (1932*a*, *b*) found that mosquito larvae could utilize colloids in suspension. To test whether this material was being used by *Daphnia* the colloids were removed from Berkefeld fitrates of Banta's culture medium by means of an ultra-filter. In this method the liquid is forced through collodion membranes by air pressure, care being taken to prevent contamination by way of the air.

Sterilized one-day-old *Daphnia* were placed in individual cotton-stoppered flasks containing 70 cc. of this ultra-filtrate and kept in an incubator at 24° C. Any flasks found at the close of the experiment

not to be sterile were eliminated just as before. Besides flasks containing sterilized *Daphnia* in untreated sterile Berkefeld filtrates of Banta's culture medium, controls were run (1) in which a collodion membrane was placed in each flask to show whether the membrane added anything to the solution which might affect the development of the *Daphnia*, and (2) in which the colloids were washed back into the flasks by reversing the collodion membranes and passing part of the ultra-filtrate again through the ultra-filter. Since none of the animals placed in Berkefeld filtrates from which the colloids had been removed attained the second molt (Table V), we may conclude that

TABLE V

EFFECT ON DEVELOPMENT OF *Daphnia* OF REMOVING THE COLLOIDS FROM BERKEFELD FILTRATES OF BANTA'S CULTURE MEDIUM

Medium	No. of Tests	Percentage of Daphnia Attaining Second Molt
Berkefeld filtrate	24	100
Berkefeld filtrate passed through ultra-filter	16	0
Berkefeld filtrate containing collodion membrane	5	100
Berkefeld filtrate ultra-filtered and then passed through filter with membranes reversed	6	100

the colloids are necessary for the development of *Daphnia*. In addition, further proof is obtained that of the two components of the (Berkefeld) filter-passing material it is the colloids and not the substances in solution which serve as a source of food for *Daphnia*.

Although it has been shown that colloids play a significant rôle in the nutrition of *Daphnia*, it is important to note that the presence of the particulate food of ordinary culture media brings about a more rapid development. Furthermore, *Daphnia* fail to produce more than one brood of young in media devoid of ordinary particulate matter. Colloids alone are therefore not suitable for nutrition indefinitely and probably act merely as a supplementary food under natural conditions. However, Krogh (1931) found in the surface waters of Lake Fureso 0.06 mg. per liter of organic nitrogen in colloidal form, 0.09 mg. per liter in the plankton, and 0.52 mg. per liter in solution. In this case it appears that the colloids present almost as large a source of food as the plankton. In small ponds

colloids were found to comprise about half the organic matter present in the water. Thus it appears that while more and more experiments are showing that dissolved substances are not used for food by aquatic animals in significant amount, contrary to Pütter's theory, organic matter in colloidal form must be taken into account more carefully in any consideration of the nutrition of these organisms.

SUMMARY

To test Pütter's theory for *Daphnia magna* sterilized animals were placed in certain culture media rendered sterile by autoclaving and by Berkefeld filtering. Although a larger proportion of the animals reached maturity in the autoclaved media, a considerable number were found to be able to utilize filter-passing material for food. This material consists of substances in solution and of colloids. *Daphnia* placed in solutions of various organic substances failed to attain the second molt. Experiments in which a solution of glucose was caused to flow through a chamber containing large numbers of *Daphnia* revealed no significant uptake of glucose by the animals. However, the utilization of the colloidal part of the filter-passing material was demonstrated by the fact that after the removal of the colloids by means of an ultra-filter *Daphnia* failed to attain the second molt.

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CONTROL OF SEX IN CLADOCERA

VIII. FOOD AND CROWDING AS FACTORS IN MALE PRODUCTION[1]

(One figure)

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THE history of experimental work on sex control in daphnids has been a search for some simple factor the presence or operation of which would cause a given female to produce male instead of the usual female young. No one factor has been shown to exercise sole control of male production in these animals. Rather, the proportion of males among the total parthenogenetic young of a group of mothers is due to the simultaneous action of at least three external agencies susceptible of experimental control, namely, the degree of crowding of the mothers, the amount of available food, and the temperature. Possibly the pH of the culture medium also may be a factor.

It is possible, as first shown definitely by Stuart and Banta (1931), to effect a degree of sex control by varying the concentration of bacteria in the culture medium. In order to obtain well-marked results by this means, it is necessary to vary the amount of available food through a wide range, a range of the order of 100 to 1. This type of experimental control of sex has been amplified and extended by Stuart and his students in several recent papers (see Literature Cited). The observed effect produced by the bacteria on the sex ratio has been assumed by these writers to be in accordance with the number of bacteria available as food for the mothers, except that, while successive dilutions of concentrated food favor male production, beyond a certain limit the further dilution of food already weak favors female production. The effect of crowding of mothers

[1] From the Department of Genetics, Carnegie Institution of Washington.

on male production has been known for a somewhat longer time, having been observed with *Moina* under experimental conditions by Grosvenor and Smith in 1913. Banta and Brown have confirmed these early observations for a number of species of Cladocera (1923*a*); and for a single species, *Moina macrocopa*, to which this paper especially refers, they have extended this line of experimental sex control beyond its original limits (Banta and Brown, 1929*a*, 1929*b*, 1929*c*).

Since both crowding and the amount of food have been shown to control male production in some manner, it is the purpose of the present paper to present and discuss data indicating the relative part each may play under given conditions.

EXPERIMENTAL

Two kinds of experimental attack were completed. Each type of experiment, while different from the other in detailed set-up, involved the same fundamental principle, namely, the effect of crowding of mothers at different food concentrations on the sex ratio of their young. In the first type of experiment crowding of the mothers and differences in the amounts of food were obtained in the following manner. The experiments were set up in differently sized but similarly shaped containers which received 75, 50, 30, 20, 10, 7.5, and 2.5 milliliters of the same medium. The amounts of surface of the medium exposed in the different containers were substantially proportional to the different volumes. The basis of the medium was a filtrate of spent culture medium which had aged for a period of from 10 days to 2 months. Bacteria presumably divide very little (when at all) in such a spent medium. This filtrate was distributed in the different containers in amounts 1 ml. less than the amounts to be used. To each of these amounts was added 1 ml. of a bacterial suspension (*Aërobacter aërogenes*) of the desired concentration. A brood of vigorous young from a single *Moina* mother was distributed into the series of containers, two to each container (in some experiments three to a container). Each experiment was really a series in which six broods and sets of containers were utilized. In addition to varying the volume of the medium, the amount of

TABLE I

DATA SHOWING THE EFFECT OF CROWDING (BY DECREASING THE VOLUMES OF CULTURE MEDIUM, WITH CONSTANT NUMBER OF MOTHERS) ON MALE PRODUCTION AND BROOD SIZE AT FOUR DIFFERENT FOOD CONCENTRATIONS

Volume Medium in Ml.		Dilutions of Added Bacteria				Summary for Crowding
		1/15	1/25	1/50	1/100	
75	No. mothers releasing	69	44	22	20	155
	Total young	1,057	555	370	133	2,115
	Av. young per mother	15.3	12.6	16.8	6.7	13.6
	No. males	157	92	15	37	301
	Percentage male young	14.9	16.6	4.1	27.8	14.2
50	No. mothers releasing	71	47	18	21	157
	Total young	1,012	638	286	129	2,065
	Av. young per mother	14.3	13.6	15.9	6.1	13.2
	No. males	281	173	73	45	572
	Percentage male young	27.8	27.1	25.5	34.9	27.7
30	No. mothers releasing	67	42	18	16	143
	Total young	991	566	238	66	1,861
	Av. young per mother	14.8	13.5	13.2	4.1	13.0
	No. males	287	176	72	17	552
	Percentage male young	29.0	31.1	30.3	25.8	29.7
20	No. mothers releasing	66	36	17	11	130
	Total young	834	426	202	35	1,497
	Av. young per mother	12.6	11.8	11.9	3.2	11.5
	No. males	270	183	88	13	554
	Percentage male young	32.4	43.0	43.6	37.1	37.0
10	No. mothers releasing	58	40	18	9	125
	Total young	764	527	223	38	1,552
	Av. young per mother	13.2	13.2	12.4	4.2	12.4
	No. males	350	287	113	16	766
	Percentage male young	45.8	54.5	50.7	42.1	49.4
7.5	No. mothers releasing	66	41	16	7	130
	Total young	842	484	186	23	1,535
	Av. young per mother	12.8	11.8	11.6	3.3	11.8
	No. males	444	271	95	10	820
	Percentage male young	52.7	56.0	52.5	43.5	53.4
5	No. mothers releasing	60	40	15	0	115
	Total young	670	470	181	.....	1,321
	Av. young per mother	11.2	11.8	12.1	.....	11.5
	No. males	469	216	81	.....	766
	Percentage male young	70.0	46.0	44.8	.....	58.0
2.5	No. mothers releasing	50	39	20	3	112
	Total young	437	415	186	9	1,047
	Av. young per mother	8.7	10.6	9.3	3.0	9.3
	No. males	265	252	113	4	634
	Percentage male young	60.6	60.7	60.8	44.4	60.6

TABLE I—*Continued*

Volume Medium in Ml.		Dilutions of Added Bacteria				Summary for Crowding
		1/15	1/25	1/50	1/100	
Summary for food	No. mothers releasing	507	329	144	87	
	Total young	6,607	4,081	1,872	433	
	Av. young per mother	13.0	12.4	13.0	5.0	
	No. males	2,523	1,650	650	142	
	Percentage male young	38.2	40.4	34.7	32.8	

food added also was varied in four steps from a strength of 1/15 dilution of a standard bacterial suspension to 1/25, 1/50, and 1/100 of the same suspension. The amounts of available food were thus varied between limits having a ratio of 6.7 to 1. At each of these four food strengths crowding of the mothers was accomplished by varying the volume of the culture medium as indicated above, so that the females were in effect crowded between limits which bear a ratio of 30 to 1. Obviously, the object was to have constant total amounts of food (in four strengths) with different volumes of medium.

Sixteen such experiments were started. In three the animals died or failed to produce young. That these failures were due to toxic properties of the bacterial suspension used was indicated by the facts that in each case the failure involved each of the different broods of animals used and that in blanks (containers to which no bacteria were added to the spent filtrate), which were run concurrently, the animals mostly remained alive but, as in all such blanks, produced few young, usually none at all.

It is not feasible to give the detailed data for all these experiments, each of which (1) was in effect a series of six experiments utilizing six broods of experimental animals and six sets of containers, and (2) involved two to four food concentrations. Table I and Figure 1*A* give the combined results of the thirteen completed experiments. The male percentages for the totals given in the last horizontal line of the table indicate the effect due to differences in the total quantity of food, since the degree of crowding remained constant. It will be seen that the male percentages due to this fac-

tor are of the same order of magnitude, although the decline in the sex ratio from 40.4 per cent males for the 1/25 dilution to 32.8 per cent males for the 1/100 dilution may indiçate a response to food, which will be discussed later. When the total quantity of food is held constant and the volume of the medium is varied (last vertical

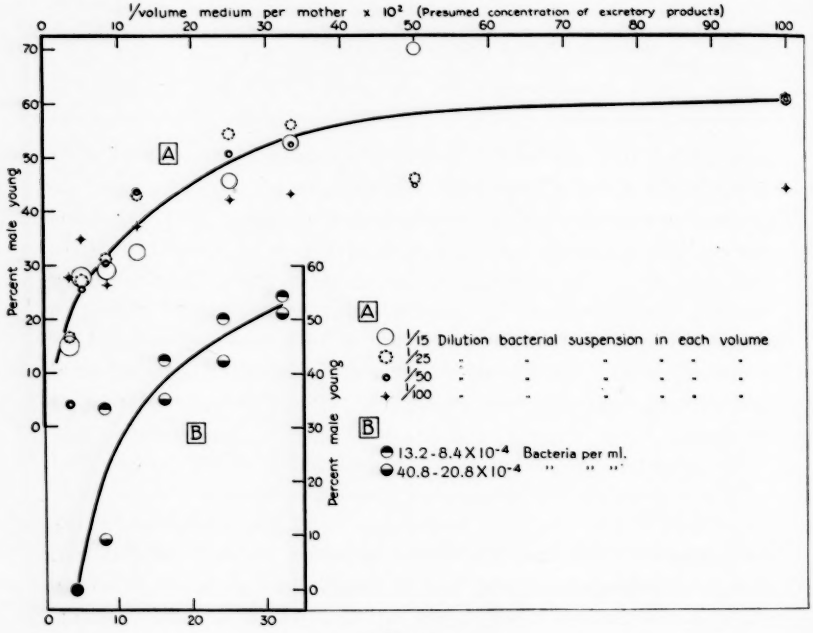

FIG. 1

column, Table I; upper curve, Fig. 1), a decided response in the sex ratio is at once apparent. For a volume of 75 ml., 14.2 per cent of the young are males. From this value the percentages of males steadily increases with decreasing volumes of culture medium (that is, increasing concentrations of by-products of metabolism) to 60.6 per cent males at 2.5 ml.[2]

[2] It is obvious that with approximately identical total quantities of bacteria in the different volumes of medium the density of bacteria in the 75 ml. volume is very much less than in the smaller volumes. It might seem that in the volumes having a lower concentration of bacteria the animals would encounter difficulty in feeding, as com-

The data for the second type of experiment on the particular rôles of the amount of food and the degree of crowding in male production are given in Table II and Figure 1*B*. Crowding in this case was ac-

TABLE II

DATA INDICATING THE EFFECT OF CROWDING ON MALE PRODUCTION AND BROOD SIZE AT TWO SOMEWHAT DIFFERENT RANGES OF FOOD CONCENTRATIONS

Date, 1931	Exp. No.	No. Mothers per 25 Ml. Culture Medium																			
		1				2				4				6				8			
		Mothers Releasing	Females	Males	Bacteria per Ml. $\times 10^{-4}$	Mothers Releasing	Females	Males	Bacteria Ml. $\times 10^{-4}$	Mothers Releasing	Females	Males	Bacteria Ml. $\times 10^{-4}$	Mothers Releasing	Females	Males	Bacteria Ml. $\times 10^{-4}$	Mothers Releasing	Females	Males	Bacteria Ml. $\times 10^{-4}$
Lower Concentrations																					
8/18	28	1	19	0	11	2	34	0	16	4	65	0	14.5	6	89	31	7.5	8	72	84	9.0
8/18	30	1	20	0	21	2	18	0	8	4	78	0	10.5	6	42	67	17.0	7	46	89	7.7
8/20	33	1	22	0	16.6	1	23	0	21.5	4	49	39	14.7	6	29	99	11.1	7	50	77	14.0
8/23	36	1	24	0	17.8	2	47	0	10.5	4	0	76	7.8	6	22	66	4.3	8	28	42	4.2
8/23	37	1	20	0	6.4	2	5	39	7.7	4	64	0	6.3	6	56	19	7.1	8	110	0	6.5
8/26	38	1	15	0	5.3	2	0	37	13.5	4	46	18	6.1	6	78	19	2.6	8	51	60	4.4
8/26	39	1	21	0	8.1	2	26	16	7.5	4	0	81	8.5	5	53	40	7.4	8	48	79	10.7
9/4	47	...	...	...	...	2	28	0	20.6	4	31	30	11.8	6	27	57	10.0	8	29	83	12.4
Av. young per mother			20.1				18.2				18.0				16.9				15.3		
Percentage male young			0.0				33.7				42.3				50.1				54.2		
Av. bacteria per ml. $\times 10^{-4}$			12.3				13.2				10.0				8.4				8.6		
Higher Concentrations																					
8/20	34	1	22	0	26.1	2	48	0	23.5	4	37	53	7.3	6	76	58	16.8	8	83	62	20.1
8/29	42	1	20	0	50.5	2	17	19	69.0	4	58	19	35.5	6	116	0	15.0	8	132	18	35.5
8/29	43	1	17	0	59.5	2	41	0	39.0	4	86	0	35.5	6	88	28	36.5	8	114	44	47.0
8/31	44	1	21	0	49.0	2	44	1	21.5	4	24	60	9.5	6	61	68	9.0	8	17	154	8.0
8/31	45	1	20	0	19.0	2	45	0	30.5	4	27	24	29.0	6	1	117	30.5	8	52	88	28.5
9/4	46	...	...	...	...	1	15	0	....	4	57	0	16.7	6	71	30	16.9	7	29	81	28.5
Av. young per mother			20.0				20.9				18.5				19.8				18.6		
Percentage male young			0.0				8.7				35.1				42.2				51.1		
Av. bacteria per ml. $\times 10^{-4}$			40.8				36.7				22.3				20.8				27.9		

pared to the volumes where the density of bacteria was greater. However, the quantity of available food seems, at most, probably a very minor factor in this experimental set-up, since the sex ratio in the different volumes is practically the same regardless of whether 1/15 or 1/100 of a standard suspension of bacteria is introduced into the different volumes. This is also indicated by the uniformity of average brood size for the different volumes (except in 1/100 concentration and in 2.5 ml. volume).

complished directly by rearing one, two, four, six, and eight mothers in the same volume (25 ml.) of manure infusion. Food strength, while the same in all vials of any one experiment at the time the mothers were placed in the medium, was determined by direct bacterial counts for each vial at the critical period for sex (Banta and Brown, 1929*c*). These counts of the density of bacteria enabled us to divide the experiments into two groups on the basis of the amount of food available to the animals, and compare experiments run with moderately low food concentrations (Fig. 1*B*, circles with top half shaded) with those having an appreciably higher food concentration (Fig. 1*B*, circles with bottom half shaded). These bacterial counts also reveal the effect the increased crowding of the mothers has on an initial food strength. The bacterial counts made at the critical period for sex indicate that the numbers of bacteria were irregularly somewhat less as the numbers of mothers increased. The most easily noted feature of Table II and Figure 1*B* is that at each of two food concentrations the male percentages increase as crowding increases. The effect of the amount of available food is also shown, since each degree of crowding (except in the case of one mother per vial) gives a higher percentage of males in the weaker food than in the food of greater concentration. But it is quite obvious that the effect of crowding on the sex ratio is notably more marked than that of the quantity of bacteria.

The similarity of the shapes of the average curves for male production in the two types of experiments (Fig. 1) clearly indicates that the same factor is responsible for the appearance of a particular proportion of males among the total young whether crowding is accomplished by increasing the number of mothers in a constant volume or by decreasing the volume of culture medium with a constant number of mothers. The common factor in the two cases seems to be the volume of culture medium for each mother.

DISCUSSION

In a series of six papers by Stuart and co-workers (see Literature Cited) which have appeared since 1931 the part played by the amount of food (bacteria) has been painstakingly investigated. The

first paper (Stuart and Banta, 1931) showed that when the amount of food available to pre-adult *Moina* females is varied through a sufficiently wide range the proportion of male young among the first clutches produced by these mothers varies in a regular manner. They showed that when the food is sufficiently concentrated mothers produce only female young; that when the food is diluted, male young appear; and that the proportion of males increases as the food becomes less concentrated, except that extreme dilution tends to lower the percentage of males.[3] Published experiments by Stuart and his students have strengthened this conclusion but have added few new facts relating to the general problem of sex control in this species. For example, Stuart, Tallman, and Cooper (1931*a*) show again that several mothers reared together in the same bottle responded in the same manner to decreased food as had the fewer mothers per bottle reported in the first paper. They also found, as would be expected, that if sufficient food was added in the form of a concentrated suspension of bacteria, male production to a great extent would be suppressed. However, in this connection it is interesting to note that the average number of young produced by the mothers receiving the added bacteria was not increased. In a third paper, Stuart, Tallman, and Cooper (1931*b*) reported that mothers excessively crowded could also be induced to produce mostly female young if, as before, sufficient food was added. A fourth paper (Stuart and Cooper, 1932) indicated that isolated (not crowded) mothers in very dilute food will at times produce males, thus confirming the observations of the first paper that food, if sufficiently different from normal, may exercise a control of the sex ratio. The fifth and sixth papers will be referred to a little later in this discussion. These four papers taken as a unit make one important contribution to the study of male production in *Moina*, namely, that the amount of available food acts as a general or limiting factor. If the food is extremely concentrated, mothers will produce female young regard-

[3] A possible confirmation of this effect of extremely weak food is shown by a comparison of male percentages (Table I) at food strengths 1/50 and 1/100 with food strength 1/15.

less of other environmental factors which may be present. On the other hand, if mothers are reared in culture media so inadequately populated with bacteria that they produce but few young, the young that are produced are apt to be female. Between these two extremes is a range of food concentrations where male young are produced with comparative ease.

As the present writers have found during their many years' work on sex control in cladocerans, it is extermely difficult to explain all data in terms of one variable in the environment. A factor which at first appears to be the key to the entire problem gradually loses some of its charm as additional experiments are completed. Perhaps this shift in emphasis is nowhere better illustrated than in the change in opinion regarding the efficacy of food as a factor in sex control (and the importance assigned to other possible factors) as expressed by Stuart and co-workers in their recent papers. The first paper (Stuart and Banta, 1931) states: "Sex of the young produced by experimentally *crowded* [italics ours] *Moina macrocopa* mothers can be controlled by the amount of available food (bacteria) present in the medium." The second paper (Stuart, Tallman, and Cooper, 1931) concludes with the statement: "Under the experimental conditions designated, food, rather than excretory substances,[4] is favored as the effective factor in sex control." While in the first two papers it was assumed that the increased male production found in the presence of crowding was due to less food for each mother so crowded, the third paper (Stuart, Cooper, and Tallman, 1931) states in conclusion: "Evidence in support of other [than excretory substances] physiological effects of animal aggregation [crowding] is pointed out." And also it is stated in this same paper: "It is concluded that conditions exist under which neither excretory products nor the quantity of available food can be ascribed as the immediate factor in the control of sex in *Moina*."

The fourth paper (Stuart and Cooper, 1932) concludes with a statement indicating an increasing doubt and uncertainty as to

[4] Excretory products, associated with crowding, have been assumed by the present writers to be a factor in male production (Banta and Brown, 1929*b*).

food as a factor and a statement limiting the type of excretory substances, viz., "Under the conditions specified in this paper the sex of the young produced by crowded mothers must be due to a lack of food together with any other effects that might be caused by diluting the medium in which the animals are reared, or to any effects caused by crowding other than the accumulation of the *transitory* and *unstable* excretory substances described by various writers." In the fifth paper (Stuart, Cooper, and Miller, 1932), while the writers are still dubious about excretory products as a sex-controlling factor, they admit, as it seems to us quite correctly, that food and temperature are factors. But this simple conclusion is now qualified when they say: "Conditions seem to exist under which the sex of the young to be produced by a cladoceran mother is not determined by the animals' immediate environment." This last conclusion had been foreshadowed in this and previous papers, in the text of which many and diverse factors have been used to explain results not readily explained by quantity of food. For example, (1) a "food accessory factor" had been mentioned (Stuart, Tallman, and Cooper, 1931, p. 591; Stuart and Cooper, 1932, p. 89); (2) possible effects of a lytic principle in the water were postulated (Stuart, Tallman, and Cooper, 1931*a*, p. 587); (3) possible "periods of depression" in the cladoceran stock (Stuart, Cooper, and Tallman, 1931, p. 601), during which mothers produce only female young regardless of the amount of food or other factors, were discussed; (4) previous "physiological injury" was assumed (Stuart, Cooper, and Tallman, 1931, p. 601) as resulting in male production, a condition which is quite independent of the immediate environment; (5) unexplained effects of aggregation affecting perhaps the activity of the mothers, which in turn affects the rate of metabolism (Stuart and Cooper, 1932), were discussed as a factor; and (6) there was the assumption (Stuart, Cooper, and Miller, 1932) of hereditarily determined "sex-fast" individuals, on which environmental influences had no effect. Mothers are apparently "sex fast" for femaleness most of the time but occasionally "sex fast" for maleness. This "hereditary" condition may last, according to these writers (p. 336),

for several successive broods of the mother but is not necessarily transmitted to her parthenogenetic young!

While perhaps there are factors, other than those recognized as amenable to experimental control, which influence the sex ratio of young *Moina*, it seems unnecessary, to the present writers, to invoke such a multitude of problematical explanations to account for what appears to us as perhaps the manifestation of a normal and usual variability such as is always present in biological material.

The sixth and most recent paper to come to our attention (Stuart, Cooper, and Coady, 1933) is of interest, giving, as we suppose it does, the current opinion of these workers regarding sex control in *Moina macrocopa*. Lack of uniformity of results when carbon dioxide is used as a male suppressing agent is explained in terms of uncontrollable "periods of depression" in the stock animals. These authors feel that there is an intimate connection between such "depression periods" and male production, and their discussion of the causes of "depressions" is summarized by the statement: "Lack of food, accumulation of waste products and alterations of the hydrogen-ion concentration of the medium have been advanced as possible causes of depression periods in Cladocera, but it is probable that many other factors are associated with this phenomenon."

Except for the inclusion of food as one of several factors which can, under certain conditions, control sex, we fail to see how these workers have clarified the problem of sex control in *Moina*. We have welcomed the doubt which the work of Stuart and students cast on the rôle of excretory products as a causative agent in control of sex, as it has helped focus attention on this phase of the work and showed that more critical experiments, such as are reported in this paper, were necessary. On the other hand, the statements made by these same workers indicating several supposed types of supra-environmental influences on the sex ratio have, we are sure, only obscured the problem.

Quantity of food as an environmental agent influencing male production belongs with temperature (Brown and Banta, 1932) as a general or limiting factor. Crowding of the mothers, as shown

definitely by our own experiments published in 1923, and again in 1929, and confirmed by the experiments reported in this paper, and as indicated by Stuart and his associates (Stuart and Cooper, 1932, pp. 86–89), seems to be the factor which is ordinarily responsible at any given temperature and food concentration (barring either extreme in the amount of food) for controlling male production. It seems to us that the evidence presented from the two types of experiments given in this paper indicates that male production is proportional in a general way to the degree of crowding of the mothers, but that the slope of the curves obtained in this way (see Fig. 1*B*) depends in part on the amount of food. Apparently a slight change in the numbers of mothers in a constant volume of medium or a change in volume of medium with constant total amounts of food produces consistent and marked alterations in male production, while published data on the amount of food with constant numbers of mothers indicate that the food must be extensively changed in order to cause corresponding changes in the sex ratio (Stuart, Tallman, and Cooper, 1931, Table I; Stuart and Banta, 1931, Table II and Fig. 3).

There is no general agreement among workers in this field as to the manner in which crowding of cladoceran mothers acts to increase male production. The present writers are of the opinion (Banta and Brown, 1929*b*), fostered by negative evidence concerning other possible factors, that unidentified and presumably unstable excretory products produced by crowded mothers are responsible for the occurrence of males. We assume that the maximum concentration of excretory products will be a function of the number of mothers and the volume of the medium, so that the reciprocal of the volume per mother can be used as a co-ordinate in representing male production graphically (Fig. 1). We assume further that quite diverse mechanical and chemical manipulations of the crowded mothers will lower the concentrations of such substances below this maximum. Statements by Stuart and his co-workers (1931, 1932) have indicated that this interpretation may be open to question. They have found, to be sure, that crowding is associated with increased male produc-

tion under conditions which eliminated food and other factors as elements, and they have offered in a recent paper (Stuart, Cooper, and Miller, 1932, pp. 338–39) the suggestion (among others) that excretory products may be responsible for increased male production by affecting the food; they also make the suggestion that metabolic products (the result of crowding) may act indirectly by causing "depressions" (Stuart, Cooper, and Coady, 1933).

There is no doubt that Cladocera do produce by-products of metabolism other than carbon dioxide and that these products are present in the culture medium. It would seem that an increase in such substances is the most probable result of the aggregation of these animals. Excretory products which are produced might be assumed to be inert, exerting no influence at all. They might, as Stuart and Cooper (1932) suggest, act indirectly through some effect on the bacteria which serve as food for the *Moina* mothers; it is conceivable, also, that the bacteria, when extremely concentrated, might destroy these products. It is probable, however, that the excretory products might affect the animals directly by producing some alteration in the protoplasm and blood stream of the mothers, which in turn affects the sex of her young. This assumption of a direct action of the excretory products on either the mother or her ovarian eggs seems to be favored by direct experimental evidence. As shown by Banta and Brown (1923, 1929*b*) and confirmed by Stuart, Cooper, and Miller (1932), aëration of crowded mothers (reared at near the optimum food concentration), through shaking or through transfer to fresh medium, usually results in a reduced male production by mothers crowded previous to such treatment. Agitation of the medium by shaking probably causes an increase in the available food through a redistribution of such food. But such an increase, as judged by direct bacterial counts which we have made is slight—too slight to cause any appreciable change in the sex ratio of young produced by the mothers reared in such shaken medium.

A pronounced reduction in male production can be accomplished under conditions which can produce very little change in the medium and comparatively little agitation of the mothers (as compared to

experiments where either the medium or the mothers are shaken) and at the same time provide excellent temporary aëration of the mothers. In the type of experiment referred to, the mothers are carefully removed from the culture bottle in a minimum of medium and placed on a microscope slide for a period of 1–3 minutes. This treatment need only be done near the time of the critical period for fixation of sex (Banta and Brown, 1929*c*) to be effective. In recent experiments thirty broods of young females divided equally between test and control, both reared in crowded bottles, gave the following male percentages among their young: aërated mothers, 10.5 per cent males; unaërated controls, 44.9 per cent males. These percentages are based on 3,428 and 3,455 young, respectively. We feel that these concise results can be explained most successfully by assuming that the aëration of the mothers on a slide affords a temporary relief from accumulated excretory products such as are present in the bottle from which they are removed. Whatever conditions are produced in the mothers by such products are quickly lowered below the male-inducing threshold; and, on the return of such mothers to their original bottles, insufficient time remains before fixation of sex for a male-inducing condition to again reach a threshold value.

The experiments of Stuart, Cooper, and Miller (1932) in which *Moina* mothers or the media in which they were reared were shaken provide an interesting confirmation of the part aëration (relief from the excretory products of crowding) plays in male production. They started bottles in triplicate, each containing five females. One of the bottles served as a control, as neither the mothers nor the medium was disturbed. These mothers gave 34.9 per cent males among their young (577 mothers). From each of the other two bottles the mothers were removed and placed temporarily in fresh bottles of medium, thus affording them a certain degree of aëration. One of the groups of mothers so transferred was further aërated by violently shaking the bottle containing them. Following this treatment, both groups were returned to their original bottles. The mothers moderately aërated by transfer to new medium produced 23.1 per cent males among their young, while the mothers aërated still further by shaking produced only 5.1 per cent males. In another

series of experiments, described in the same paper, the mothers, by means of a divided tube, were held undisturbed in the bottom of the tube while the top four-fifths of the culture medium was removed and shaken. This lack of disturbance of the mothers resulted in no alteration of the sex ratio when they were compared to competent controls (16.0 per cent males and 16.6 per cent males, respectively).

The final experiment reported in this paper (Stuart, Cooper, and Miller, 1932, Table IV) affords another example of the limiting effect of the amount of available food: when the food is greatly reduced, female production is indicated (Stuart and Banta, 1931; Stuart, Tallman, and Cooper, 1931*a*). Working with bottles having so sparse a flora of bacteria that the average number of young per mother was reduced to 7.1, these workers were able, by shaking, to raise the average number of young per mother to 10.2, an increase of 44 per cent. Accompanying this change in the amount of food, probably because of increased oxygen tension and the redistribution of food, the male percentage rose from 17.4 to 29.5 per cent (in accordance with the interpretation that at a very low food level mostly female young are produced, while at a less low food level male production occurs).[5]

Of the various elements in the environment which have been shown by numerous workers to exercise a control of the sex ratio in cladocerans, four have received most attention. These are: (1) the relation of the number of mothers to the volume of the culture medium (i.e., isolation and crowding experiments); (2) the concentration of food (bacteria or algae) in the culture medium; (3) the temperature; and (4) the pH of the medium. The last of the foregoing has as yet been insufficiently investigated to enable us to draw definite conclusions (Banta and Brown, 1929*b;* Tauson, 1930; Stuart, Cooper, and Coady, 1933). The effect of different temperatures on the sex ratio presents no particular complexity (Brown and Banta, 1932). In the case of crowding and food experiments, however, con-

[5] The usual size of first broods as reported by these authors in other experiments varied around fourteen. The relationship which exists between the number of young produced and the sex ratio at different food strengths will be discussed in a later paper.

trary interpretations regarding the causal agent in male production are possible, since the introduction of increasing numbers of mothers into a limited volume of culture medium not only increases the concentration of excretory products of the daphnids but also in general tends to reduce the number of bacteria present. In the present paper we have attempted to explain and compare the specific rôles played by food and crowding. To us the data given and referred to indicate that the quantity of food exercises a control near the limits of its usable range. The limits of its range are, of course, death of the cladocerans from starvation at low concentration and death from toxic conditions due to the bacteria at high concentrations. These lethal concentrations bear a relation to each other of the order of about 1 to 10,000.

Since within the food range just mentioned brood size does not vary with increasing concentration of bacteria in the same manner as male production, it is well to call attention briefly to each separately. Brood size seems to be a function of the concentration of bacteria only when the culture medium is quite sparsely or very densely populated with bacteria (Stuart, Tallman, and Cooper, 1931, Table I). An increase in concentration of bacteria in the former case (relatively few bacteria) results in larger broods of young, while further increases in a concentration already high results in a smaller average number of young per mother. Between these two extremes in the amount of available food, the brood size of mothers is particularly insensitive to difference in the food supply. Apparently, other factors determine maximum brood size when sufficient bacteria are present.

Concentration of bacteria as a factor in male production on the contrary is noticeable only when the concentrations are below the optimum for maximum brood size. Even here the result of decreasing food is not uniform, as it is in the case of brood size, since a fall in the concentration of bacteria first results in increased male production; but, if continued, results in decreased male production.

Superimposed on these broad effects is the effect of crowding (see discussion of data presented in Table I and Fig. 1). As the volume

of medium per mother is lowered (increasing concentration of excretory products), there is a corresponding rise in male production. And it is significant that these effects are produced without markedly altering the quantity of food or the average size of broods of the mothers.

SUMMARY

1. Data are presented which show the relative rôles of crowding of the mothers and the amount of available food as sex controlling factors in *Moina macrocopa*.

2. From these data and the pertinent literature it appears that the amount of food acts as a broad or limiting factor in male production, while crowding acts as a direct or immediate factor.

3. Data presented and discussed suggest that the excretory products produced by crowded mothers act directly to increase male production.

4. The dependence of brood size on the concentration of bacteria is discussed. The gradient in numbers of young per brood is not coincident with the gradient for percentages of males produced.

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THE ORIGIN AND EARLY DEVELOPMENT OF SOMATIC BEHAVIOR IN THE ALBINO RAT

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THE studies on development of behavior in the cat, which have been reported from this laboratory (Windle and Griffin, 1931; Windle, O'Donnell, and Glasshagle, 1933; Windle, 1934*b*; Windle, Orr, and Minear, 1934), have led to some results and conclusions which appear to be at variance with those of investigators using different animals and experimental methods. Although this is particularly evident in the work on rat embryos reported by Angulo (1932), it was thought that the differences are more apparent than real. Consequently, it seemed desirable to make an adequate test of conditions in the rat with methods comparable to those employed in investigating behavioral development in the former animal. One is placed in a position to evaluate the observations of others working in this field only by acquiring first-hand knowledge of the sequence of events in embryonic and fetal life of several forms of animals. Such, at least, is the opinion of the present authors and constitutes the main reason for diverging from an intensive investigation of behavior of the cat.

MATERIAL AND METHODS

Animals from two sources were used in the present experiments. One group of ordinary white rats of unknown ancestry was obtained from a local dealer. Another group was obtained from the Wistar Institute; the age of the latter specimens was known and they were kept under feeding and breeding conditions as nearly as possible like those prevailing at the Wistar Institute. These rats served as controls, in a sense, for the ones of unknown stock; and the use of them assured that material was comparable in every way with that used by others for similar experiments.

[1] The senior author directed and took part in all experiments and is responsible for composing this report.

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Litter ages were computed from accurately recorded matings to the time of opening the abdomen for examination of the embryos. Embryos were measured at the end of each experiment, a variable interval of time after delivery, while the specimens lay in dishes of physiological saline solution. The crown-rump length of a living embryo varied slightly during the course of experiments; some were slightly shorter when first removed from the amnion than an hour later. Consequently, tendencies toward pseudo-accuracy have been avoided by expressing sizes of most specimens in steps of half a millimeter.

At the beginning of this series of studies on the rat, an attempt was made to use Swenson's (1925) method of ligating carotid arteries and jugular veins to render the pregnant animals unconscious before examining the embryos. However, it was found that we were faced with the necessity either of giving ether during the study of the embryos or else practicing vivisection on struggling and apparently conscious rats. After satisfying ourselves that an anesthetic administered to the mother does affect the movements of her embryos, the procedure was discontinued and a new one better suited to the purpose was devised. Other investigators (e.g., Hooker and Nicholas, 1930) have realized the desirability of avoiding ether anesthesia during studies on embryos. In fact, Swenson (1931) himself discarded the first method in favor of a new one, for this reason. Our procedure has been described in detail elsewhere (Windle and Minear, 1933). We ligated the carotid arteries and their collateral branches in the neck, and the basilar artery through the base of the skull. This completely interrupted blood flow to all parts of the brain rostral to the medulla oblongata and produced the typical decerebrate state in the rat. The procedure is a delicate one, and many animals had to be discarded; nevertheless, an equal number survived and maintained a good condition throughout an hour or more of experimentation on the embryos. When the operation was performed properly, practically no blood was lost (sometimes only a trace on one 3-mm. sponge), respirations became rapid and deep, and the rat lay quietly on the table with legs and tail extended.[3]

[3] Unless the basilar artery was tied nearly as far rostrad as the caudal border of the pons, true decerebrate rigidity failed to develop.

Precautions were taken to conserve body heat during the operation but, even though the animal was blanketed with cotton, the rectal temperature nearly always dropped. This soon returned to normal when heat was applied over the head and chest. It was found inadvisable to apply heat to the abdomen; when this was done, some of the embryos were nearly always found dead. In half an hour or more, after the body temperature had been restored and all effects of the ether anesthetic had disappeared, the rat was placed on a small operating board and immersed in a constant temperature (38° C.) bath of physiological saline solution.

Methods of experimentation on the embryos varied considerably, but, in general, the following procedure was followed. One vesicle of the uterus was opened and the embryo freed from its membranes. While the circulation was intact,[4] various forms of stimulation were applied to the surface of the specimen. Especial note was taken of responses which followed flipping the forelimbs with a stiff fiber probe (cactus spine). Sometimes, faradic stimulation of various parts was employed.[5] Frequently after these preliminary experiments *in situ*, the umbilical cord was ligated, effects of this procedure were recorded, and the specimen was placed in a small dish of warmed saline solution where detailed experimentation could be performed more conveniently. Responses to mechanical stimulation were again studied and exploration with faradic stimuli was continued. In some experiments, mechanical stimuli and, in others, faradic shocks were the main ones used.

Throughout, an attempt was made to work as rapidly as possible because it had been observed in the cat experiments (Windle, Orr, and Minear, 1934) that early reflexes were often seen only during the initial moments after delivery of the embryos. Many of the present experiments were not prolonged beyond 10 minutes for each individual specimen. At least two observers took part in each experiment, and a stenographer was employed to record results.

[4] After expelling a rat embryo, the uterine wall contracted on the placenta; it is doubtful if placental exchange was perfect after this had happened.

[5] Shocks were delivered by the secondary coil of an inductorium (3 volts on primary) withdrawn 5–9 cm. Electrodes were fashioned of fine nichrome wire with points less than 0.5 mm. apart.

OBSERVATIONS AND DISCUSSION

The present experiments are concerned principally with the development of function of skeletal-muscular and nervous systems in the rat during the sixteenth and seventeenth days (374–400 hours) after insemination. It is realized that the study is incomplete in some respects and that, with our more recent developments of the technical procedures, it can be extended profitably. However, sufficient information was obtained to indicate that early behavioral development of the rat is similar to that of the cat in its general aspects and differs in only a few particulars.

Throughout this series of experiments, we tried to determine accurately what each specimen was capable of doing, to record factual observations objectively, and to avoid attempting interpretations of behavior in the light of theories of development until data had accumulated. We would emphasize the point that our interest lay in the determination of behavioral capabilities. There is a great difference between the normal behavior of an embryo, i.e., what it actually does *in utero*, and its behavioral capabilities, i.e., what it can be made to do immediately after removal from the uterus. To determine normal behavior, one would have to limit himself to observations of spontaneous movements; even then, he could be certain that conditions are not like those *in utero*. To study the capabilities, one must not be limited to any particular form of stimulation or any one method of experimentation. It is just as desirable to test the proprioceptive mechanisms of the embryo as to examine the exteroceptive ones, and it may be more instructive to learn the effects of relatively strong stimulation than to fail to obtain results with weak stimulation.[6]

DIRECT STIMULATION OF SKELETAL MUSCLE

Many of the rat embryos examined in the present experiments and which did not respond reflexly to one stimulus or another, were tested with faradic shocks to determine whether or not the muscles

[6] It is difficult to say what stimulus is strong and what weak when applied to a delicate embryo. We consider light touch with such instruments as brush, hair, fiber probe, etc., which did not displace the embryo as "light." Flipping the limbs, however gently, with a probe and applying faradic shocks produced the sort of stimulation we have called "strong."

were capable of contraction. Likewise some of those which exhibited reflexes were tested at the end of the experiment. Not every specimen was examined in this way and, unfortunately, complete records were not always kept; but in most cases, one or more representatives of a litter was explored with the micro-electrodes. Table I summarizes the results obtained on eighty-one embryos between 9 mm. and 14.5 mm. long, 350–400 hours after insemination. Specimens larger than this almost uniformly exhibited all the responses listed in Table I.

The skeletal musculature of the rat reaches a functional state before a neural reflex mechanism can activate it. The first direct muscular response was observed in 11–11.5-mm. embryos and consisted of outward and forward or backward motion of the forelimb bud. At about 11.5 mm., the head could be caused to move laterad, and soon the whole trunk even as far caudad was the tail showed motion. By the 12-mm. stage, muscle contractions moved the distal part of the limb, apparently at the embryonic elbow joint; and by 12.5 mm., at the wrist. Motion at the hip was entirely passive and occurred with trunk contractions until about 13 mm. The muscles on the side of the abdomen began to be active at 12.5 mm., and those of the tail gave responses irregularly after 12 mm. Stimulation of the facial muscles and the extraocular muscles, under a lens, produced no reaction in one 13-mm. embryo, but the eye and ear could be caused to move in two 14.5-mm. specimens. Flexion at the knee was present at 14.5 mm. We may conclude that the course of physiological muscular development proceeds, in a very general way, caudad and rostrad from the shoulder region and distad and ventrad from the dorsal part of the trunk.

Newly functional muscle is less responsive to weak stimuli than is that developed at an earlier stage. When the first responses occurred, they could be obtained only by using strong current (secondary coil withdrawn 5 cm.). By 13 mm., the forelimb would respond to weak current (coil withdrawn 7–9 cm.), but the neck and caudad part of the trunk still required a greater intensity (coil 6–7 cm.).

Several other factors seem to determine whether or not responses to direct stimulation will be observed. Embryo size is para-

mount; perhaps the age of the specimen plays a small part irrespective of size. Motility was at its best in healthy specimens kept in the constant-temperature bath. Ligation of the umbilical cord and cooling or bleeding the embryo eventually led to their abolition. However, specimens as small as 12.5 mm., which were removed from rats dying during operation and in which the heart had stopped, showed good forelimb, trunk, and neck movements. Excessive heat applied to the embryo very quickly rendered the muscle incapable of contraction, but the specimen could be cooled 10 or 15 degrees with no appreciable effect appearing for some time.

The character of the response which follows faradic shocks applied directly over a muscle mass is not like that of the early reflexes of embryos. (1) The first contractions were relatively slow, but they become more rapid with increase in size of specimen. (2) The response nearly always appeared to be maximal with the strength of stimuli used. (3) It followed immediately after the application of the stimulus, and no appreciable latent period intervened. (4) The muscle was held in tetanus and relaxed slowly. (5) One response after another could be obtained with no suggestion of a refractory phase. (6) It was distinctly localized, and the plane of movement varied closely with the position of the electrodes. (7) The physiological state of embryo and parent rat did not affect the response greatly.

The capacity of the muscles for response to direct stimulation is about the same as in the cat embryo of slightly greater length (Windle, Orr, and Minear, 1934). However, the rat of 11 mm. appears to be farther advanced in morphological development than the cat of the same size; consequently, the skeletal musculature seems to reach a functional state relatively earlier in the cat than in the rat.

REFLEXES[7]—GENERAL FEATURES

Rat embryos begin to respond reflexly to various stimuli about the middle of the sixteenth day after insemination. By the end of

[7] We hold to the theory that the movements of effectors involve conduction over an afferent neurone and a transfer in the central nervous system to the efferent neurone. The possibility that some are caused by direct stimulation of efferent neurones must be borne in mind. Reasons for our contention that movements are reflexes appear in the body of this report.

that day, flipping the forelimb produced movements of the forelimb and tapping the snout resulted in movements of the head. These responses differed from those obtained by stimulating the muscle directly and had many of the characteristics of true spinal reflexes. They were (1) relatively quicker, (2) not necessarily moving the member as far as it was capable of being moved by maximal contraction of the muscle. (3) A noticeable latent period usually appeared to follow the application of the stimulus, and sometimes summation of stimuli seemed to be effective when a single one failed. (4) There was no marked tetanic quality of the response, the member returning quickly to the normal position. (5) One response nearly always seemed to render a succeeding stimulus ineffectual for some moments, suggesting a long refractory phase. (6) The response was quite stereotyped; regardless of where the stimulus was applied, the plane of reflex movement was the same. (7) Its presence depended to a very great extent upon the physiological condition not alone of the embryo but likewise of the parent rat. These characteristics appear to be shared by the forelimb, head, trunk, and hindlimb reflexes in their earliest development.

It is realized that any method of stimulating the delicate structures of small embryos is really extremely crude. But it was only by using rather strong stimuli over a relatively great area that first responses could be obtained at all. In the preceding statements a qualitative difference between the so-called "reflex" and the response to direct faradization is implied. We believe that the response we call a "reflex" is a quick incomplete contraction because a weak impulse reaches a small part of the muscle mass through newly functional nervous centers; whereas the direct response is strong and tetanic because the strong direct stimulus spreads into a large mass of muscles unmodified by conduction through the spinal cord.

The latent period which follows a stimulus and precedes the response was of relatively great magnitude—in fact, was perceptible to the eye. The implication is that there is a long delay in the central nervous system in the case of the reflex. The muscle itself possesses a latent period, but it is apparently not so great because one could observe no interval between application of stimulus to the muscle directly and the response.

Although we are far from certain of the presence of the phenomenon of summation (the factor of coincidence probably plays a part), the refractory phase appears to be quite real. Almost never, in the youngest specimens, could a second reflex of head or forelimb be obtained immediately after one response. The refractory period seemed to become shorter with advance in size and age of embryos.

That the condition of the parent determines the presence or absence of reflexes is demonstrated in Table II and appended statements. Here are listed all animals which survived the examination of at least one embryo. All successful experiments are marked in the table by an asterisk. It should be observed that in practically every one of these ligation of the basilar artery was performed with no blood clot forming around the brain stem; consequently, respirations were strong, deep, and often more rapid than normal. The embryos obtained from these animals were in excellent condition, free from effects of an anesthetic, and almost uniformly showed many reflex phenomena.

The experiments not marked with an asterisk were ruled out of major consideration because of (1) poor health of rat, (2) rupture of basilar artery with clot formation on the brain stem producing shallow, irregular, or slow respirations, (3) ether given during observations on the embryos, or (4) some unknown factor causing death of the rat before observations were completed. We believe that we can be reasonably certain of observing most of the responses of which the embryos are capable only by selecting the experiments in which we know the rats to be in excellent physiological condition. Table II bears out this conclusion.

The molding of the response by the position of the stimulus, so characteristic in direct stimulation of the embryonic musculature, is not a feature of the reflex movements of rat embryos. Although the plane of head movement can be controlled to a large extent by placing the micro-electrodes in various positions over muscles of the neck, most stimuli applied to the snout caused contralateral head flexion accompanied by extension if the stimulus was strong. There can be no question that these movements were reflexes, because the contracting muscles were some distance from the point of application of

the stimulus and on the opposite side of the body. Although direct faradic stimuli produced movements of the forelimb in a number of planes, flipping the limb (no matter what direction) resulted in movement of but one type—an abduction (involving distal segments of the limb) with backward movement of the forelimb. This same response was sometimes superimposed on a forward motion of the limb caused by direct stimulation of the muscles on the rostral border of the limb. Furthermore, the same thing appeared in some specimens after stimulating the caudal end of the trunk dorsal to the base of the tail. Such stimulation likewise at times produced the head extension. With these facts in view, there can hardly be more question of the reflex nature of the forelimb response than there is of that of the head.

THE FORELIMB REFLEXES

Some rat embryos between 12 mm. and 13 mm. long and 374–84 hours old (copulation age) responded to mechanical stimulation of the forelimbs. When the limb was flipped with a fiber probe, there followed a quick twitch of the limb outward and backward. This movement had the characteristics of a reflex and resembled similar reactions observed in the cat and chick (Windle, Orr, and Minear, 1934; Orr and Windle, 1934). In the smallest specimens, it appeared to involve the entire limb distal to the trunk, but it is doubtful if any movement of trunk accompanied it. The distal joints were clearly involved in this response in larger embryos 13–15 mm. long. Sometimes the reflex was of smaller magnitude than at other times, and was then often confined to the wrist or elbow joints.

Although pressure or stretching provided adequate stimuli for forelimb reflexes in embryos smaller than 14 mm., faradic shocks applied to the tips of the digits or even light tactile stimuli occasionally elicited responses in ones 14–15 mm. long. All embryos (thirty-six specimens) 14+–15 mm. long and twenty-six of the thirty-eight between 13 mm. and 14 mm. long responded to mechanical forms of stimulation, but only eight of the forty less than 13 mm. long did so.

Exactly the same type of forelimb movement frequently followed strong faradic stimuli applied to the back caudal to the forelimbs.

All but one of the twelve embryos 14+–15 mm. long, five of the eleven specimens between 13 mm. and 14 mm., and five of the fifteen between 11.5 mm. and –13 mm. which were so tested exhibited this reaction. In the smallest embryos, the electrodes had to be placed over the spinal cord just caudal to the forelimbs; but in the others, the response could be obtained by stimulating any point over the spinal cord from the level of the forelimbs to that of the hind limbs or tail. This probably indicates a progressive growth caudad of functional neural mechanisms.

The response appeared to be truly reflex and not caused by directly stimulating the muscle; a local contraction of the trunk musculature in the neighborhood of the electrode points could nearly always be observed, and this was repeated with every shock. But the reflex came after this initial local movement and a latent period intervened between the reflex and the initial local movement; the response could seldom be repeated until an interval of time had elapsed. In short, the reaction had the qualities of other reflexes.

How do these observations on the reflexes of the forelimb compare with those of others who have studied rat embryos? Angulo (1932) originally made no mention of having used stimuli comparable to ours, and reported independent forelimb movements in no embryos smaller than 15.7 mm. and in very few less than 18 mm. More recently, Angulo (1933*a*) described, very briefly, experiments with stronger mechanical stimuli but did not give sizes of embryos. On the other hand, Swenson (1926) reported many forelimb movements in specimens smaller than 15 mm., but apparently they were usually associated with trunk or neck reflexes. Nevertheless, he describes one experiment in which he bent back the forelimb of a 14-mm. embryo and obtained the forelimb response, and others in which touching the forelimb caused limb twitches. There is little question that these were like the reflexes we report. It is our opinion that others have failed to see forelimb reflexes because they did not stimulate the specimens adequately. We have shown them to be characteristic of such widely different forms as cat (Windle, Orr, and Minear, 1934) and chick (Orr and Windle, 1934).

Coming, as it were, on top of all the physiological evidence of the

reflex nature of the early forelimb movements, are correlated anatomical studies on the development of the first reflex arcs in the spinal cord. We do not propose to consider them in detail in this article. They were reported before the American Association of Anatomists meeting in Philadelphia in March of 1934 (Windle, 1934*a*). Briefly, the development of reflex arcs goes hand in hand with the development of the forelimb reflexes. The last link in such arcs are the collateral branches of sensory posterior funiculus fibers. These are incompletely formed in specimens from which we could elicit no foreleg reflexes and well developed in those which showed the phenomena. Surely, it is not merely coincidence that we should have discovered the anatomical mechanisms for the first local spinal reflexes in those chicks (Windle and Orr, 1934) and cats (Windle, 1934*b*) and now those rats from which we have been able to obtain the first reflex behavior.

THE REFLEX NECK AND TRUNK MOVEMENTS

A few embryos smaller than 14 mm. long exhibited reflex movements of the head or upper trunk when the snout was stimulated; the smallest was 12 mm. long. In this specimen and in another of 12.5 mm. the response was a flexion of the head toward the side from which the stimulus was applied, accompanied by a movement backward. In all other instances stimulation resulted in movement of the head toward the opposite side. Movement resulted from contraction of the neck muscles without involvement of the trunk and shoulder in any of these small embryos. All reflexes were obtained with faradic stimulation, and persistent effort was not made to obtain them by mechanical means. They were much more easily elicited with strong stimuli than with weak ones; faradic shocks were effective before touch.

During the course of development the snout reflex grows or expands, as it were, so that by 14–14.5 mm. the movement not only involved neck musculature but also that of the rostral part of the trunk in the region of the forelimbs. Consequently, accompanying each strong lateral flexion or extension of the head, the forelimbs moved at the shoulder backward and forward with contraction of trunk muscles. This was at first purely a passive motility, but it

soon became active although not separate from trunk contractions. It must not be confused with the usual forelimb reflex following stimulation of the limbs, because the latter involved distal limb joints whereas the former movement seemed to be accomplished almost entirely at the shoulder and with the trunk. It was frequently observed that, by the 14.5–15-mm. stage, when limbs move uniformly with neck and trunk extension, a weak mechanical or faradic stimulus still produced simply a contralateral head flexion without extension; whereas a strong shock would throw the whole rostral end of the embryo into a marked extension.

Reflex movements of the rostral part of the trunk or neck could sometimes be obtained by stimulation of the back at the caudal end of the embryo. Thus, in two 12-mm. and one 13-mm. embryos, stimulation over the spinal cord caudal to the forelimbs elicited quick extension of the trunk in the region of the forelimbs. Head extension was seen in two of these. The movements appeared to be reflex in nature and followed the local contraction at the site of stimulation after definite latent periods. Such reactions were more frequently noted in larger specimens; eleven of the eighteen embryos 14–15 mm. long showed head or rostral trunk reflexes after stimulating the back in front of the tail, but only three of the twenty-three less than 14 mm. long that were tested did so.

How do the present observations on development of neck and trunk reflex movements compare with those made by other investigators? Although we have used different kinds of stimuli and different experimental methods, the results are essentially the same as those reported by Swenson (1926) and Angulo (1932). The former studied but a few (twenty) embryos smaller than 14 mm. long and obtained these reactions in four specimens of 13.5 mm. (20 per cent), using faradic and mechanical stimuli. The latter examined ninety-seven[8] embryos smaller than 14 mm. and obtained trunk or neck movements in twenty-four of them (25 per cent). We tested sixty-six specimens less than 14 mm. long and found trunk or neck reflexes in thirteen (20 per cent); it should be noted that 41 per cent of ours

[8] Seven were not measured, but we assume them to have been less than 14 mm. long because their litter mates varied from 12.6 mm. to 14 mm.

were less than 13 mm., whereas only 30 per cent of Angulo's were less than 13 mm. long. Nearly all of Swenson's specimens 14–15 mm. long showed neck and trunk reactions; forty-five of Angulo's sixty embryos 14–15 mm. reacted (75 per cent); and forty-two of our fifty specimens of like size gave similar responses (84 per cent). For the whole series 15 mm. or less in length, we obtained reactions in 48 per cent; Angulo found them in 44 per cent; and Swenson, in 68 per cent. Therefore, there can be no doubt that reflex movements of the rostral portion of the trunk and neck make their appearance in the last half of the sixteenth day after insemination in rat embryos 12–15 mm. long.

RELATIONSHIP BETWEEN TRUNK AND LIMB REFLEXES

The reflexes of the neck and trunk have been considered separately from those of the forelimb because there appears to be no relationship between the two in early stages. The trunk reflexes obtained by stimulating the snout constitute the so-called "mass reaction." The early forelimb reflexes seem to be entirely unrelated to this reaction. Perhaps mass reaction is not the most satisfactory term to use, because it may imply a response which is maximal. In embryos of the present series, light stimulation of the snout with a brush or hair often produced contralateral head flexion, and sometimes extension as well; whereas strong stimulation nearly always gave both responses, and in addition more frequently brought trunk and shoulder muscles into play than did lighter stimuli. With increase in size of specimens, more and more muscles become activated in massive trunk responses. But in very few of the present embryos did stimulation of the snout set off movements in the distal part of the forelimbs, even though these structures were involved in local responses.

The local forelimb reflexes appeared, if not actually before the trunk reflexes, at least with greater consistency in the younger and smaller specimens. In a few instances, the forelimb twitches were followed by reactions of the neck or trunk rostral to the forelimb, but only in the larger members of the series. It is somewhat surprising that they did not set off these massive trunk movements more

often, because conduction undoubtedly goes rostrad in the nervous system even in the smallest specimens. This was indicated by the results of stimulating the caudal end of the embryos with faradic shocks. Stimuli so placed were followed by a homolateral series of reflex-like responses which usually occurred in the following order: (1) lateral flexion of trunk at or rostral to shoulder, (2) backward and outward twitch of forelimb, (3) extension of the head or, in 15-mm. embryos, extension of head and trunk down below the shoulder and movement of the limb at shoulder with this extension. Thus the forelimb twitch became integrated with other reflexes in an ascending chain but took no part in the descending "wave" of activity which constitutes the mass reaction.

One must consider the possibility that responses in the rostral part of the body resulting from stimulating more caudally may not have been reflexes in the strict sense. They may be explained in several ways: perhaps they resulted from conduction up ascending pathways in the spinal cord stimulated directly. If such were the case, at least two and probably three neurones, with one or two synapses but no sensory receptors, were involved. An alternative explanation is that a local contraction of the musculature, stimulated directly by the electric current, may have set up a chain of proprioceptive reflexes by pressure on adjacent myotome derivatives, as was suggested by Hooker and Nicholas (1930) for a similar phenomenon of behavior in young rats after spinal-cord section *in utero*. The former theory seems the more reasonable in the present instance because responses were obtained only with strong stimuli; even though weak ones caused a local contraction of muscle, which should have been as efficient a stimulus for a proprioceptive ascending chain of reflexes as those induced by stronger ones, no reflex-like reactions followed. Perhaps a detailed study of the anatomical structure of the nervous system of these embryos will shed some light on the question.

Other observations tend to indicate that there is a large degree of independence in the earliest responses to stimulation. The forelimb reflex was more persistent, as a rule, in embryos less than 14 mm. long than was the snout reflex; after ligating the umbilical

cord or allowing a specimen to cool, the latter were more quickly abolished than the former. However, this relationship soon underwent a reversal and in 15–16 mm. embryos one usually found that the neck and trunk reactions had become the predominant ones and outlasted the forelimb responses under conditions leading to death of the specimens. These observations help to explain the fact that we observed only foreleg reflexes in a number of experiments (some of those not marked with asterisks in Table II) in which we knew the parent rat to be depressed in some way.

SPONTANEOUS MOVEMENTS AND RESPONSES AFTER LIGATION OF THE UMBILICAL CORD

Spontaneous movements, that is, those appearing after no intentional stimulation, were seldom observed in specimens less than 15 mm. long, while the placental circulation remained intact. Movements were induced in a few of this size or smaller and in specimens larger than 15 mm. by tying the umbilical cord. They were exceedingly irregular in occurrence and usually appeared in the forelimb, the trunk, or in both; they were obtained with greater regularity in 18-mm. specimens than in the small ones.

Efforts were made to determine the effect of cord ligation on the reflex movements that could be obtained with circulation intact in our selected series of experiments. In a few instances, reflexes could be induced with greater ease and regularity immediately after ligating the cord, but nearly always disappeared entirely in a very few minutes. In most cases, ligation of the umbilical cord did not facilitate the observation of reflexes which had been seen with intact circulation; on the contrary, this procedure abolished all responses in a few minutes. As was indicated previously, all of them did not disappear at the same time; usually the snout reflex, with its trunk movements, dropped out before the response of the forelimb to flipping the limb. In the few instances in which reflexes seemed to be enhanced by ligation of the cord, it seemed to be the forelimb reaction that was benefited most.

Some embryos were not studied for long periods of time with the placental circulation intact. It is impossible to say that reflexes could not have been obtained had time been taken for prolonged

studies. Specimens could be subjected to much closer examination after complete removal from the uterus, where respiratory movements of the mother no longer interfered; therefore some of them were removed after very brief or no examination *in situ* with the hope that we might discover some behavioral reactions present only in initial moments after delivery, such as was the case in the cat embryos. These procedures were justifiable in the present experiments because we were primarily concerned, not in determining the behavior that normally takes place, but in finding out what systems can be made to function (in other words, the capabilities of the specimens) regardless of the methods used. In these, forelimb and snout reflexes that did not make their appearance before ligation were sometimes obtained after tying the umbilical cord.

Some of the embryos which did not exhibit spontaneous behavior immediately after tying the cord, and others which did so, likewise showed another type of behavior. After these specimens had been tested in various ways, they were often laid aside in small dishes of saline solution and allowed to cool to room temperature. Others were allowed to bleed by severing the umbilical cord. Not infrequently, slow vermiform movements appeared in the musculature of the trunk and forelimbs which caused the embryos to flex very slowly or caused the limbs to wave slightly. Similar movements were observed in cat embryos. It seems reasonable that they were manifestations of death of the specimens by cooling or by the sudden withdrawal of blood from the muscle or nervous system. The responses appeared within from 1 to 5 minutes after bleeding, regardless of the length of time elapsing between tying the umbilical cord and cutting it. In other instances, they appeared 20–50 minutes after tying the umbilical cord and before cutting it; but in these, the embryos had been allowed to cool to room temperature or were in a dying condition. These results differed from those on cat embryos in which we observed what appeared to be a closer relationship between delivery, ligation of cord, and bleeding (Windle, Orr, and Minear, 1934).

Angulo (1930, 1933*b*, 1934) described results of tying the umbilical cord in rat embryos 16–19½ days old. He reported (1930) that "if the umbilical cord of any fetus is ligated, it will recapitulate the

maximum degree of physiological activity it has achieved, but in the inverse order of its normal development." However, he did not obtain the forelimb reflexes comparable to those seen by Swenson and by the present investigators and, consequently, formulated his law of behavioral development on incomplete observations. The present observations do not support his statement that these movements occur in the inverse order of normal development because we have demonstrated that forelimbs move in response to stimuli as soon as, or possibly even before, the massive neck and trunk responses if we consider only experiments with circulation intact. It appears to more nearly fit the facts now available that responses following ligation of the umbilical cord in Angulo's experiments simply recapitulated behavioral capabilities, some of which had not been observed before cord ligation because adequate stimuli were not used to elicit them.

INTERPRETATIONS

The present series of experiments indicate that somatic behavior patterns make their appearance and start their development in the rat very much as they do in the cat. There can be no doubt that the somatic musculature becomes functional, not all at once, but progressively after an expanding pattern, as it were. The pattern may not expand in a perfectly even, regular fashion; but, nevertheless, one sees a more or less gradual acquisition of excitability caudad, rostrad, and distad from the locus of first appearance. This is quite to be expected, because we know that morphological development proceeds in a similar fashion.

Development of reflex responses appears to follow the course of expansion of the motor excitability pattern imperfectly. Obviously, reflexes cannot occur until muscles can be activated; but there are other factors, such as the development of sensory and motor endings and connections in the central nervous system, which must determine the presence or absence of reflexes; and we cannot be certain that all of these invariably mature in the same order as the muscles. Nevertheless, there is a pattern of reflex behavioral development comparable to that of the muscular system, inasmuch as reflexes of the fore part of the body become active before those of

the hind part and reflexes of the proximal part of limbs precede those of the distal segments. The behavioral pattern is an expanding one, in a sense, more and more muscles functioning as the embryo grows. However, one must question whether the individual movements we have observed arise by a process of breaking-up or individuation from a completely integrated primary behavior pattern as Coghill (1929) finds them in *Amblystoma*. The fact that the local twitch of the forelimb outward and backward does not occur with movement of the head and trunk in the early stages, but is entirely independent of these, would seem to speak for a type of behavior different from that in *Amblystoma*.

In the snout reflex we believe we see a pattern of behavior expanding caudad by the acquisition of more and more functional myotome groups. In the forelimb reflex we believe we find a similar, but distally expanding, pattern. The integrating of these two must remain unexplained for the time being, but we believe that it is not necessary to have a co-ordinating mechanism in the central nervous system for sequentially integrated movements to take place. This has been demonstrated for older rat fetuses and newborn rats by Hooker and Nicholas (1930).

SUMMARY

The skeletal musculature of rat embryos began to respond to faradic shock applied directly to it when the specimens had attained a length of about 11–11.5 mm. The first excitable muscle was that in the region of the primitive shoulder. During the course of development, excitability spread rostrad and caudad, as well as distad and ventrad, from this region.

Some embryos 12–13 mm. long began to respond reflexly to various types of stimuli about the middle of the sixteenth day after insemination. Flipping the forelimb at times induced a quick outward and backward twitch of the limb as a whole or of its distal part, depending upon the size of the specimen. This response was usually unrelated to any trunk movements. Stimulating the snout with faradic shocks, or with mechanical agents in larger specimens, at times induced a quick extension of the head with flexion toward the opposite side. During the course of development this response

spread caudad and involved more of the trunk muscles, then the shoulder muscles passively, and finally the shoulder muscles actively. This expanding snout reflex, the mass reaction, was independent of the local forelimb reflex which nearly always confined itself to the distal forelimb joints at the stage in which the mass reaction involved the shoulder.

Reflex-like movements of the forelimb, trunk, and neck could be elicited at times by stimulating specimens dorsal to the spinal cord at points caudal to the forelimbs. These responses occurred in a caudocephalic series; and, in some of the larger embryos of this series, the mass reaction (cephalocaudal activity) followed such a series of movements.

All reflexes seemed to require strong stimuli administered over relatively large areas in their incipiency. Light forms of stimulation became adequate at a later time. All reflexes possessed a number of characteristics, such as latent period, refractory phase, and stereotyped pattern, that differentiated them from the responses of muscle stimulated directly. Furthermore, they depended to a much greater extent upon the physiological condition of embryo and parent rat than did the direct responses.

Some inconclusive experiments on the effects of ligating the umbilical cord seemed to indicate that, after reflexes had become well established, such a procedure exerted a transient stimulating effect upon them. However, at the time of first appearance of the reflexes, ligation of the cord usually seemed to have little effect except to abolish them entirely.

Peculiar, vermiform contractions of muscles already activated in reflex responses sometimes appeared at the conclusion of experiments in which specimens had been bled or cooled. The significance of these is not known.

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NOTE ADDED MARCH 27, 1935

An important article by Leonard Carmichael, bearing upon the question of developing of behavior, appeared in Vol. 16 of Genetic Psychology Monographs. Its title is "An experimental study in the prenatal guinea-pig of the origin and development of reflexes and patterns of behavior in relation to the stimulation of specific receptor areas during the period of active fetal life." The stand taken by the present investigators against the concept of individuation of reflexes from a total behavior pattern in the cat and rat is in agreement with the observations and conclusions of Carmichael.

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TABLE I

RESULTS FROM DIRECT STIMULATION OF EMBRYONIC MUSCLE*

LITTER		EMBRYO		LOCAL MOVEMENT ELICITED AT:									
No.	Age (Hr.)	No.	Size (Mm.)	Fore-limb	Neck	Trunk	El-bow	Hind-limb	Tail	Ab-do-men	Wrist	Knee	Eye Ear
12:34	350	2 embryos	9–9.5	−	−	−	−	−					
11:34	377	3	11	+	−	−	−	−					
19:33	368	3	11+	+	−	−	−	−					
19:33	368	7	11.5	+	−	−	−	−					
72:33	376	2 embryos	11.5	+	−	−	−	−					
32:34	374	4	11.5	+	+	+	−	+	−	−	−		
18:34	377	1	−12	+	+	−	−	−	−	−	−		
19:33	368	2	12	+	+								
31:34	370	2 embryos	12	+	−	?	?	−					
32:34	374	6	12	+	+	+	+	+	+	−			
32:34	374	9	12	+	+	+	+	+	+	−	?		
224:33	375	5	12	+	−			−					
11:34	377	1, 2, 5	12	+	+	+	−	−					
9:33	378	4, 5	12	+	−	−							
14:34	380	3, 4	12	+	+	+	+	−	−	−			
20:34	381	2	12	+	+	+	+	−	−	−	−		
19:33	368	4	12+	+	+								
9:33	378	9	12+	+	−	−	−						
32:34	374	1, 2	12.5	+	+	+	+	+	−	+	+		
32:34	374	3	12.5	+	+	+	+	+	+	+	+		
224:33	375	6, 7, 8	12.5	+	−	−	+	−					
212:33	377	1, 2	12.5	+	+	+	+		+				
9:33	378	2, 6	12.5	+	−	−	+						
22:34	378	2	12.5	+	+								
14:34	380	1	12.5	+	+	−		−	−				
14:34	380	2	12.5	+	+	+	+	−			?		
235:33	377	1	−13	+	+	+	+	+	+				
9:33	378	10	−13	+	?		−						
19:33	368	1	13	+	+	+		+					
31:34	370	1 embryo	13	+	+	+		−	−				
32:34	374	8	13	+	+	+	+	+	+				
72:33	376	1 embryo	13	+	+	+		+		+			
18:34	377	2	13	+	+	+	+	+	−	+	+		
18:34	377	6	13	+	+	+	+	+	−	−			−
94:33	378	2	13	+	+	+	+						

* Age is expressed by the nearest hour after mating. The first movement of the forelimb involved the musculature of the limb at the point of attachment of the limb. The first movement of the hind limb involved trunk muscles rostral to the limb and was therefore "passive."

TABLE I—*Continued*

Litter		Embryo		Local Movement Elicited at:									
No.	Age (Hr.)	No.	Size (Mm.)	Fore-limb	Neck	Trunk	El-bow	Hind-limb	Tail	Ab-do-men	Wrist	Knee	Eye Ear
6:34	379	1	13	+	+	+	+	+					
27:34	381	2 embryos	13	+	+	+	+	+					
16:33	383	1	13	+	+	+	+	+	+				
19:33	368	5, 6	13+	+	+	+	+	+					
29:33	377	3	13.5	+	+	+	+	+		+			
16:33	383	2	13.5	+	+	+	+	+	+				
27:33	384	1	13.5	+	+	+	+	+	−	+			
6:34	379	2, 3	14	+	+	+	+	+	−	+	?		
6:33	381	5	14	+	+	+	+	+					
234:33	382	4	14	+	+	+	+	+					
36:34	383	2, 3	14	+	+	+	+	+	+	+	+	?	
16:33	383	3, 4	14	+	+	+	+	+	+				
22:33	384	5	14	+	+	+	+	+	+	+	+		
10:34	386	5	14	+	+	+	+	+	+	+			
18:33	388	1, 3	14	+	+	+	+	+	+	+	+		
7:34	380	1	14.5	+	+	+	+	+	+	+			
13:34	380	3	14.5	+	+	+	+	+	−				
22:33	384	2, 4	14.5	+	+	+	+	+	+	+	+		
10:34	386	6 embryos	14.5	+	+	+	+	+	+	+	+		
12:33	389	2 embryos	14.5	+	+	+	+	+	+	+	+		
19:34	400	5, 6	14.5	+	+	+	+	+	+	+	+	+	+

TABLE II

SUMMARY OF EXPERIMENTS IN WHICH ANIMALS SURVIVED OBSERVATION OF ONE OR MORE EMBRYOS

Line No.	Animal		Operative Conditions							Embryos				
	Rat No.	Age (Days)	Anesthetic	Time (Min.)	Blood (Cc.)	Respiration	Temperature (Degrees F.)	Recovery Time (Min.)	Decerebrate Rigidity	Litter Age (Nearest Hour)	Size (Mm. C.R.)	Reflexes: Forelimb	Reflexes: Neck	Selected Experiments
1	19:33	120–50	AD	30	2		94–99	55	no	368	11+–13+	–	–	
2	25:33	120–50	AD		?			0	no	368	12	–	–	
3	31:34	133	AD	25	5	slow		5	no	370	12–13	–	–	
4	32:34	179	AD	20	trace	184–280	95.2–98.6	55	marked	374	11.5–13	+	+	*
5	224:33	110–30	AD	30	trace	good	?–97	54		375	12–13	–	–	*
6	72:33	130–50	AD	30				30		376	11.5––13	–	–	
7	29:33	130–50	AD	30	trace	fast regular	98–99	63	marked	376	12.5–13.5	+	+	*
8	235:33	110–30	AD	30	1	normal regular	97–97	68	slight	377	12+–13	+	–	*
9	18:34	180	AD	25	1	144–160	95–98.6	60	good	377	12––13	+	–	*
10	11:34	165	AD	30	6	weak	?–97	15	no	377	11–13	–	–	
11	212:33	120–50	AD	30	3	irregular	98–107	76	no	377	11.5–12.5	–	–	
12	34:33	110–30	AD	30	trace	regular	?–97	64		377	12–13.5	+	+	*
13	9:33	120–40	AD	35	trace	200	94.4–97	65	slight	378	12––13	+	–	*
14	94:33	110–30	AD	30	2		97–101.6	45	no	378	12.5–14	–	–	
15	22:34	163	AD	30	trace	152	95.6–99.7	49	marked	378	12–12.5	–	–	
16	6:34	159	AD	25	4	regular 40		0	no	379	13–14	–	–	
16[1]							97	54	no		13.5	+	–	
17	30:34	176	AD	30	trace	Cheyne-Stokes	95.6–98.4	33	no	380	13–15	–	–	
18	3:34	161	AD	25	5	weak		30	no	380	14–14.5	–	–	
19	14:34	186	AD	35	trace	76–140	94–101.8	57	no	380	12–12.5	+	–	

20	13:34	134	AD	25	1	regular	95–95.4	41	no	380	14–15	+	−	*
21	161:33	110–40	CL	10	trace	normal		20	no	381	12–14	+	+	*
22	20:34	207	AD	30	trace	178–200	95.2–99	45	marked	381	12–14	+	+	
23	6:33	110–40	CL	10	trace	normal		0	no	381	14–14.5	+	−	
24	16:34	166	AD	25	1.5	104–112	96.8–101.4	50	slight	381	14–15	+	+	*
25	234:33	110–40	AD	30	1	good	?–97	58		382	12.5–15	+	+	*
26	3:33	110–30	CL	10	trace	normal		0	no	382	14–15	+	−	
27	23:34	143	AD		trace		97	57		382	?	−	−	
28	36:34	178	AD	30	trace	108–164	97.6–101.4	75	no	383	13.5–14.5	+	+	*
29	1:33	110–30	CL	10	trace	normal		20	no	383	13.5–15	+	+	*
30	16:33	110–40	AD		6	poor		0		383	13–14	−	−	
31	11:33	110–40	CL	10	trace	normal		20	no	384	12.5–14.5	+	+	*
32	22:33	110–40	AD	25	trace	good	96–100	70	no	384	13.5–14.5	+	−	
33	24:34	164	AD	25	5	slow		20	no	384	14	−	−	
34	28:34	185	AD	25	trace	80–160	97.2–100.6	59	no	384	13–14.5	−	−	
35	2:33	110–30	CL	10	trace	normal		20	no	384	14–15	+	+	*
36	4:33	120–50	AD	25	trace	134	95.4–99	40	present	384	14.5–15	+	+	*
37	4:34	152	AD	25	trace	108	95.8–99.6	95	marked	384	14.5–15	+	+	*
38	2:34	140	AD	30	4	weak	97	56	no	385	15.5	−	−	
39	10:34	164	AD	30	trace	180	96.4–101.4	31	good	386	14–14.5	+	+	*
40	18:33	110–40	AD	30	trace	slow irregular	93–99	30	no	388	14–15	+	−	
41	9:34	167	AD	35	3	slow weak	95.2–100.8	57	no	389	14–15	−	−	
42	12:33	110–40	AD	30	trace	good	91–101.4	80		389	15	+	+	*
43	19:34	196	AD	25	trace	148–180	95.6–99	55	no	400	14.5–15	+	+	*
44	20:33	110–40	AD	30	trace	regular	94–99.4	60	no	401	14.5–16	+	+	*
45	7:33	110–30	CL	10	trace	normal		20	no	405	17+–18	+	+	*
46	17:33	110–40	AD	40	1	regular	95–103	75	no	426	19–20	+	+	*

TABLE II—*Continued*

EXPLANATION OF TABLE II

Operative conditions.—Abbreviations indicate anemic decerebration (AD) and carotid ligation (CL), methods employed to produce the unconscious state; both were carried out under ether. Under "Blood" is indicated highest estimated amount of hemorrhage from all sources; where the work "trace" is used, less than 0.5 cc. is implied. The rectal temperature is given in degrees F. taken at intervals after the operation and before the animal was placed in the water bath. Recovery time was measured from the moment ether was discontinued at the time the basilar artery was ligated until the abdomen was opened to observe the embryos. Decerebrate rigidity was not always present, even in the successful operations; its development seemed to depend upon rather high ligation of the basilar artery.

Embryos.—Litter age, computed from observed mating to the time the abdomen was opened, forms the basis for arrangement of experiments in this table; figures are given in the nearest hour. For detailed consideration of reflexes refer to Table III. Selected experiments are marked with an asterisk (*); all but the last two are given consideration in Table III; and the reasons for the selection, when not evident from Table II, are given in the following "remarks."

Remarks.—The numerals refer to the line numbers of this table. (1) Blood lost from sinuses lateral to basilar artery formed clot on brain; no information on rate of respiration—probably slow. (2) Animal died early in experiment; respiratory infection evident at beginning of operation. (3) Basilar artery ruptured with formation of clot on brain; rat lived nearly an hour. (4) Operation was as nearly as possible perfect; no hemorrhage from basilar artery, and not enough blood from all sources to wet one 3-mm. sponge. All reflexes of embryos were obtained after umbilical cords had been ligated. (5) Limited use of faradic stimuli, but reflexes were definitely absent when mechanical stimuli were used and without ligating cords. (6) No record of operative conditions ruled this experiment out of consideration in Table III. (7) Operation as nearly as possible perfect; less than 1 drop of blood lost from all sources. Reflexes obtained with circulation undisturbed. (8, 9) Hemorrhage, mainly from muscle attachments, did not affect respirations. Reflexes obtained with circulation intact (8) and with it interrupted (9). (10) Basilar artery ruptured when attempt was made to coagulate it with high-frequency electro-surgical unit. (11) Hemorrhage from minute branches of basilar artery produced a clot around the brain stem. Note marked temperature rise; animal very irritable. (12) good operation; reflexes obtained before and after ligating umbilical cords. (13) Hemorrhage very slight, mainly from neck dissection. Reflexes obtained with circulation of embryos intact. (14) Rat died after three embryos had been examined. (15) Regardless of what appeared to be a perfect operation, the rat died within 10 minutes of the beginning of observations. One embryo had been examined; it was a non-motile 12-mm. specimen. (16) The basilar artery was broken, and observations were begun on the embryos before the animal had recovered from the ether; no responses from embryos. The rat's condition began to improve, respirations increased, and it was decided to allow some time for possible recovery. The last specimen was examined (16*a*) sometime later, and it exhibited foreleg reflexes with the placental circulation undisturbed. (17) Although hemorrhage was only 2 or 3 drops, the rat developed Cheyne-Stokes respiration before

the end of the experiment. Embryos had died recently. (18) The basilar artery was ruptured; rat lived less than an hour. (19) Less than 0.5 cc. of blood from neck dissection and venous sinuses; but there probably was a small clot formed intracranially; considerable serous fluid in lungs; rat died at conclusion of observations. All embryos were small; one 12-mm. specimen exhibited reflexes after ligation of cord and in response to faradic stimulation of back caudal to the forelimbs. (20) Hemorrhage mainly from dissection of neck structures. Considerable serous fluid from the lungs; animal very irritable. Reflexes obtained from forelimbs with placental circulation intact and after cord had been ligated. (21) No decerebration attempted; animal normal 20 minutes after operation. All reflexes obtained with placental circulation intact. (22) Operation as nearly as possible perfect. Reflexes obtained after ligation of umbilical cords. (23) Recovery from ether not allowed. (24) An unusually small basilar artery was encountered; this was ruptured, but hemorrhage was slight and the condition of the rat seemed to warrant including this experiment in the selected series. Reflexes obtained before and after ligating umbilical cords. (25) Hemorrhage from muscle attachments. Reflexes obtained before and after ligating umbilical cords. Experiment included in Table III but not considered as good as most. (26) Rat etherized during observations on embryos. (27) High-frequency cautery used; all embryos dead, thought to have been electrocuted. (28) Hemorrhage not more than 1 drop. Low ligation of basilar; no rigidity developed, but reflexes were lively. Most of the reflexes of embryos were obtained after ligating umbilical cords. (29) No decerebration attempted; 20 minutes allowed for recovery from ether. All reflexes obtained with placental circulation intact and with mechanical stimuli. (30) Basilar broken; no recovery from ether allowed. (31) Same as 29. Reflexes obtained before and after tying cords. (32) This rat appeared to be in good condition, but heat applied to abdomen seemed to have rendered embryos sluggish; rat died after 1 hour of experiment. Reflexes obtained before ligating umbilical cords. (33) Basilar artery broken; animal did not survive observations. (34) Operation good, but heat applied to abdomen killed embryos. (35) No decerebration attempted; 20 minutes allowed for recovery from ether; no faradic stimuli used. All reflexes present before ligation of umbilical cords. (36, 37) Operations excellent. Reflexes obtained before and after ligation of umbilical cords. (38) Basilar artery ruptured with high-frequency cautery. (39) Same as 36 and 37. (40) Rat in poor health; infection of one eye; two of five embryos were dead. (41) Hemorrhage from ruptured basilar artery. Animal very irritable, with spasms of jumping movements. Considerable serous fluid from lungs. (42, 43, 44) Same as 36 and 37. (45, 46) Experiments good but not considered in Table III because of size and age of specimens.

TABLE III

SUMMARY OF REFLEX RESPONSES OF THE FORELIMBS AND TRUNK (NECK) OF 11.5-MM. TO 15-MM. RAT EMBRYOS IN SELECTED EXPERIMENTS MARKED WITH ASTERISKS IN TABLE II

Line No.	Litter		Embryo		I. Forelimb Stimulated: Reflex Movements of—		II. Back or Tail Stimulated: Reflex Movements of—					III. Snout or Face Stimulated: Reflex Movements of—			Reflexes Appeared after Tying Umbilical Cord (Min.)
	No.	Age (Nearest Hour)	No.	Size (Mm.)	Whole Forelimb	Distal Joints	Whole Forelimb	Distal Joints	Rostral Trunk	Lateral Head Flexion	Head Extension	Lateral Head Flexion	Head Extension	Rostral Trunk	
1	32:34	374	4	11.5	—	—	F	—	—	—	—	—	—	—	2
2	18:34	377	1	—12	—	—	—	—	—	—	—	—	—	—	
3	32:34	374	6	12	—	—	F	F	F	?	F	F	F	—	1
4	32:34	374	7	12	—	—	—	—	—	—	—	—	—	—	
5	32:34	374	9	12	—	—	?	F	F	—	—	—	—	—	3
6	224:33	375	3	12	—	—						—	—	—	
7	224:33	375	5	12	—	—						—	—	—	
8	34:33	377	8	12	—	—						—	—	—	
9	9:33	378	3, 4, 5	12	—	—						—	—	—	
10	161:33	381	6	12	—	—						—	—	—	
11	20:34	381	2	12	—	—	—	—	—	—	—	—	—	—	
12	235:33	376	7	12+	—	—						—	—	—	
13	34:33	377	9	12+	M	—						—	—	—	0 0
14	9:33	378	8	12+	M	—						—	—	—	
15	9:33	378	9	12+	—	—						—	—	—	
16	32:34	374	1, 2	12.5	—	—	—	—	—	—	—	—	—	—	
17	32:34	374	3	12.5	—	—	F	?	—	—	—	—	—	—	1
18	32:34	374	5	12.5	—	—	F	F	—	—	—	F	F	—	3
19	224:33	375	2, 4	12.5	—	—						—	—	—	
20	224:33	375	6, 7, 8	12.5	—	—	—	—	—	—	—	—	—	—	

21	29:33	376	5	12.5	—	—	—	—	—	—	—	—	—	F	—	1
22	18:34	377	5	12.5	—	—	—	—	—	—	—	—	—	—	—	
23	34:33	377	2	12.5	—	—	—	—	—	—	—	—	—	—	—	0–2
24	34:33	377	12	12.5	M	—	—	—	—	—	—	F	—	—	—	
25	9:33	378	2, 7	12.5	—	—	—	—	—	—	—	—	—	—	—	
26	9:33	378	6	12.5	M	M	—	—	—	—	—	—	—	—	—	0
27	234:33	382	6	12.5	—	—	—	—	—	—	—	—	—	—	—	
28	11:33	384	6	12.5	M	*	—	—	—	—	—	—	—	—	—	0
29	235:33	377	1, 4	—13	M	M	—	—	—	—	—	—	—	—	—	0
30	235:33	377	5, 6	—13	—	—	—	—	—	—	—	—	—	—	—	
31	9:33	378	10	—13	M	M	—	—	—	—	—	—	—	—	—	0
32	32:34	374	8	13	M	*	F	?	F	F	F	—	—	—	—	3
33	224:33	375	1	13	—	—	—	—	—	—	—	—	—	—	—	
34	29:33	376	1, 4	13	M	—	—	—	—	—	—	—	—	—	—	0
35	235:33	377	2	13	—	—	—	—	—	—	—	—	—	—	—	
36	235:33	377	3	13	M	*	—	F	—	—	—	—	—	—	—	0
37	18:34	377	2	13	—	—	—	—	—	—	—	—	—	—	—	7
38	18:34	377	6	13	—	—	—	—	—	—	—	—	—	—	—	
39	34:33	377	1, 6	13	M	—	—	—	—	—	—	—	—	—	—	0
40	34:33	377	3	13	—	—	—	—	—	—	—	—	—	—	—	
41	34:33	377	5, 10	13	—	—	—	—	—	—	—	—	—	—	—	1–4
42	34:33	377	11	13	—	—	—	—	—	—	—	—	—	—	—	0
43	20:34	381	3	13	—	M	—	F	—	—	—	F	F	—	—	0
44	29:33	376	2	13+	M	?	—		—	—	—	F	—	—	—	
45	29:33	376	3	13.5	M	M	—					—	—	—	—	
46	34:33	377	7	13.5	—	—	—	—	—	—	—	—	—	—	—	0
47	101:33	381	3, 5	13.5	M	?	—	—	—	—	—	FM	FM	—	—	
48	36:34	383	4	13.5	—	—	—	—	—	—	—	—	—	—	—	
49	1:33	383	4, 10	13.5	M	*	—	—	—	—	—	—	—	—	—	0
50	101:33	381	1, 2, 4	—14	M	*						FM	FM			0
51	20:34	381	1	14	—	*	—	—	—	—	—	—	—	—	—	6
52	16:34	381	1	14	M	*	F	F	F	—	—	F	F	F	—	0
53	234:33	382	4	14	MF	*	—	—	—	—	—	F	F	—	F	0
54	36:34	383	2	14	M	*	—	—	—	—	—	—	—	—	—	1
55	36:34	383	3, 5	14	—	—	—	—	—	—	—	—	—	—	—	

TABLE III—*Continued*

Line No.	Litter		Embryo		I. Forelimb Stimulated: Reflex Movements of—		II. Back or Tail Stimulated: Reflex Movements of—					III. Snout or Face Stimulated: Reflex Movements of—			Reflexes Appeared after Tying Umbilical Cord (Min.)
	No.	Age (Nearest Hour)	No.	Size (Mm.)	Whole Forelimb	Distal Joints	Whole Forelimb	Distal Joints	Rostral Trunk	Lateral Head Flexion	Head Extension	Lateral Head Flexion	Head Extension	Rostral Trunk	
56.....	1:33	383	2, 3, 9	14	M	M						M	M	—	0
57.....	11:33	384	5	14	M	M						M	M		0
58.....	11:33	384	8	14	M	M						—	—		0
59.....	2:33	384	4	14	M	M						M	M		0
60.....	10:34	386	5	14	M	M	F	F	F		F	MF	MF	F	0
61.....	36:34	383	6	14+	—	M	—	—	—	—	—	—	—	—	2
62.....	234:33	382	1	14.5	M	M						—	—	—	0
63.....	234:33	382	2	14.5	M	*						MF	MF	F	0
64.....	234:33	382	3	14.5	M	MF						F	F	F	0
65.....	36:34	383	7	14.5	M	M	F	F	F	F	F	F	F	F	1
66.....	1:33	383	6, 7	14.5	M	*						M	M	M	0
67.....	11:33	384	3, 4, 7	14.5	M	*						M	M		0
68.....	4:33	384	3	14.5	M	M						MF	MF	F	0
69.....	4:33	384	6, 7	14.5	M	M						M	M	—	0
70.....	4:34	384	5	14.5	M	M						MF	MF	F	0
71.....	10:34	386	2	14.5	M	M	F	F	F		F	—	—	—	1
72.....	10:34	386	3	14.5	M	M	—	F	F	F	F	MF	MF	F	0
73.....	10:34	386	4, 6	14.5	M	M	F	F	F		F	MF	MF	F	0
74.....	10:34	386	7	14.5	MF	MF	F	F	F	F	F	MF	MF	F	2
75.....	19:34	400	5, 6	14.5	M	M	F	F	—	—	—	MF	MF	F	0

76.....	4:34	384	2	−15	M	M						MF	MF	F	0
77.....	1:33	383	5	15	M	M						M	M		0
78.....	2:33	384	2, 3	15	M	M						M	M	M	0
79.....	4:33	384	1	15	M	M						MF	MF	MF	0
80.....	4:33	384	2	15	M	M						MF	F	F	0
81.....	4:33	384	4	15	M	M						MF	MF	MF	0
82.....	4:34	384	1	15	M	M						−	−	−	1
83.....	4:34	384	3	15								F	F	F	1
84.....	4:34	384	6	15	M	M						F	F	F	0
85.....	4:34	384	7	15	M	M						MF	MF	MF	0
86.....	12:33	389	1, 2	15	M	M	F	F	F		F	MF	MF	MF	0
87.....	19:34	400	4	15	M	M	F	F	−	−	F	MF	MF		0
88.....	19:34	400	7	15	M	M	F	F	−	−	−	MF	MF	F	0
89.....	20:33	401	7	15	M	M						M	M	M	0

EXPLANATION OF TABLE III

In this table are listed all healthy living embryos 11.5–15 mm. long that were tested physiologically in experiments marked with asterisks in Table II. They are arranged according to length, rather than age, because their behavioral responses appear to depend mostly upon their size. Consideration has been given to the results of stimulating three regions: forelimb, back or base of tail, and snout or side of head. Space does not permit tabulation of other irregular responses; nor does it allow a complete summary of protocols. However, the authors are willing to furnish copies of their summarized protocols, in which specimens are arranged according to line number in this table, to anyone interested in a more detailed account of their results.

The letters "M" and "F" indicate presence of reflexes in response to mechanical and faradic stimulation. The question mark (?) denotes doubtful responses. The minus sign (−) indicates that tests were made but no response was obtained. Blank spaces indicate either that no tests were made or that responses were absent and no record of them was kept. Asterisks(*) in the second column of responses (distal joints) mark experiments for which we recorded forelimb reflexes without specific mention of the exact location of the movement. In most of the motile specimens, the forelimbs seemed to move at the shoulder, but no trunk movement accompanied this response; such movement is implied for the responses listed in the first column of reflexes. In the last column of the table, the length of time elapsed after umbilical cord ligation before any reflexes were obtained is indicated.

In line No. 29, stimulation of the back was applied to but one of the two specimens. In line No. 51, a reflex twitch of pelvis occurred 6 minutes after ligating cord.

THE SPECTRAL EFFECT ON THE GROWTH-RATE OF TADPOLES[1]

(One figure)

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THE relationship of light and radiation to animal growth has not been clarified or very satisfactorily investigated. There are abundant references in the literature to light-effects, but the majority of these are subject to the criticism that there was lacking a clear understanding of the physical variables involved. In most cases one variable was controlled and all others were neglected.

In respect to the frog's egg, Edwards (1824) stated that tadpoles would not develop at all in the dark. Schnetzler (1874) said that tadpole growth was slightly retarded in the dark although Higginbottom (1850 and 1863) and McDonnell (1859) had found no differential growth-rate of tadpoles in light and dark. Yung (1878) claimed that tadpoles raised in light for 30 days were 25 per cent longer than those raised in the dark, and that at 60 days this difference was reduced to 6 per cent. Finally, Corradetti (1933) stated that the larvae of *R. esculenta* develop more rapidly in black than in green or white containers. We have here a gradual but complete reversal of opinion in regard to the ability of tadpoles to grow in the dark.

It is the purpose of this study to attempt to clarify the distinction between light and radiation by the presentation of statistical data on the development of the frog's egg under different parts of the spectrum, using equivalent amounts of radiant energy.

APPARATUS AND METHOD

The constant temperature bath used consisted of a unit from a General Electric Refrigerating Machine with the cooling elements suspended in the bath and controlled by a telechrone device. The apparatus can be run constantly, but it was found best to run it for 40 seconds out of every minute, and partially to balance this with a

[1] It is a pleasure to express here my appreciation of the unlimited facilities provided for this work by the Department of Zoölogy, Columbia University.

small (100-watt) heating element measuring 18 inches in length and one-half inch in diameter, also manufactured by the General Electric Company. This heating cylinder contained an electric cord soldered within so that the heater was at the lower end, submerged nearly to the bottom of the tank but well shielded from the water. Actual temperature regulation was by means of a second and larger heater, also suspended in the tank, controlled by a mercury regulator. This regulator circuit consisted of a mercury column, a source of potential, a very high-resistance relay with large magnets of many windings. This relay operated on a very small current and ran a low resistance relay (operated at 6 volts) which made contacts through the nichrome wire and mercury for the working circuit. The tank measured $3.67 \times 1.42 \times 1.42$ feet deep. With such a large body of water, stirred with a propeller run by a constant-duty motor, the temperature distribution was so rapid that there was very little fluctuation, if any.

The light source used was a new G.E. type S1 sunlamp, suspended directly over the constant temperature bath. The radiant energy and spectral transmission through various filters combined with this lamp were measured from time to time to check the total amount of energy available and the stability of the filters. The spectral range of this lamp is from about 2600 A to a point deep in the infra-red, with almost a continuous band from about 3,000 A into the infra-red. Since it usually took about 15 minutes for the lamp to attain maximum energy output, this interval was allowed prior to radiations. During the life of a good bulb, which may be as much as 500 hours, there is considerable blackening with some increase in the relative ultra-violet output. However, the change in total energy at the various spectral regions was of no significance during the interval of a single set of observations, representing at the most about 15 hours.

The filters used in these experiments were all of Corning glass, cut to 6.5 inches square. The spectral transmissions were determined by careful spectrophotometric readings before and after the series of experiments. These filters were found to be very stable, particularly as compared with the gelatin filters when used with the powerful S1 sunlamp at rather close range. The blue, green, and infra-red filters

were pure, while the yellow and red carried a little beyond their respective limits. The transmission of these filters is shown in Figure 1.

The radiant energy transmitted through each of these filters was measured by the paired-thermometer method. A metal box measuring $5.5 \times 3.5 \times 2.5$ inches was so constructed as to act as a water chamber within which was suspended a smaller metal box measuring $3.75 \times 2.75 \times 1.5$ inches. Two high-precision thermometers were

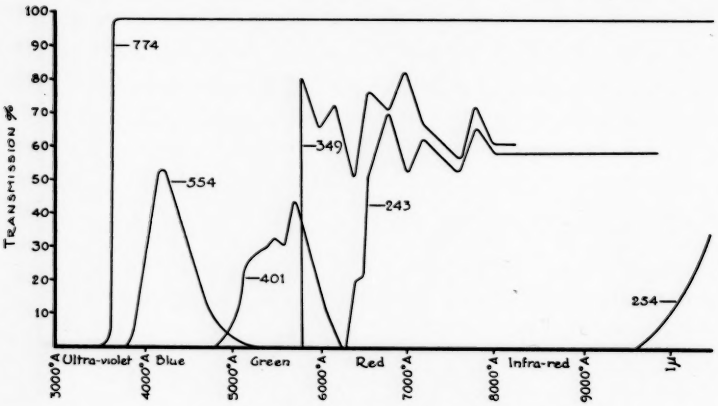

FIG. 1.—Spectral transmission of Corning glass filters

placed parallel to each other and 1.75 inches apart so that their mercury bulbs were within the inner air-tight box. One of the thermometers was exposed to the outside through a window of heat-resisting clear Corex D (Corning) glass and was blackened with optical black to a point well above the mercury bulb. This thermometer was shielded from the other by a metal partition, allowing, however, free currents of air around the two thermometers. The inner box was carefully soldered into place so that it remained air-tight but was surrounded by a constantly moving water bath. The inlet and outlet tubes for water circulation were located at one end of the larger box and at adjacent corners.

In absolute units the thermometers showed a difference of 1°C. for approximately 0.2 gm. cal. per sq. cm. per minute. Before each reading the two thermometers were brought to equilibrium in the bath and then the blackened thermometer was exposed to the source

of radiation. Mid-summer direct sunlight showed a difference of 32.30°C. between the two thermometers while direct sunlight at a corresponding time in January showed a difference of only 19.60°C. The apparatus will respond to room-light radiant energy, but with such small amount of radiant energy the thermopile method would be the more accurate. With the wide range and the large amount of radiant energy used in these experiments, the paired-thermometer method proved to be the more practical. With the blackened thermometer there was non-selective absorption of all radiant energy available, irrespective of spectral differences, and this energy was represented in centrigrade-degrees difference between the two thermometers. While the reaction time of the thermometers is somewhat longer than for thermopiles, the ultimate values are equally dependable and reproducible. The percentage transmission of radiant energy, calculated for each filter, was based upon the total radiant energy of the unshielded sunlamp as 100 per cent. The values represented for each filter are therefore relative values, relative to the total amount of energy available. The temperature differences of the two thermometers are not, however, relative, since they measure total radiant energy available through each filter. If, for instance, the difference between the two thermometers was 10.00°C. for the monochromatic red and 5.00°C. for the monochromatic green, it could be said that the radiant energy transmitted by the green filter was about half that of the red filter.

The material used was the frog's egg, secured from *Rana pipiens* in the manner recently described (Rugh, 1934*a*). The eggs were kept in the dark during the first two hours after fertilization and then were brought out into subdued light where the eggs that had just cleaved were separated from the others. A total of 105 eggs were selected at exactly the same stage of cleavage for each experiment, and were separated into seven lots of 15 each. These eggs were then placed in seven regulation finger bowls. The finger bowls had been coated on the sides and bottom with black Valspar and within each finger bowl a 5 mm. depression measuring 5 cm. in diameter was made in paraffin blackened with lampblack. This limited the area in which the eggs were placed so that all eggs would be similarly treated. During the period of radiation only sufficient spring water was added to cover the eggs. These finger bowls were suspended in

the water bath to a point just below the upper edge, and were supported by a wire basket through which water freely circulated from the constant-temperature bath. Each finger bowl was shielded from all radiations except those coming directly from the lamp. Radiant-energy readings were taken at the exact points where the groups of eggs were placed under the various filters. In this manner the amount of energy received by each group of eggs under a restricted portion of the spectrum was recorded.

Between radiations the eggs, or tadpoles, were placed in large (10-inch) finger bowls partitioned off by galvanized wire screen so that each set of eggs was in a separate compartment. Diffusion continuity equalized all other variables as recently described (Rugh, 1934*b*). These finger bowls were kept in total darkness between radiations and the water was changed daily. After the fifteenth day food, in the form of liverwurst suspension, was added. Since this food was unaffected by available light, and the number of tadpoles per compartment was the same, all of the normal variables except the controlled radiation were equalized.

The control group of eggs was treated in exactly the same way as the radiated groups, except that they were kept in total darkness. During radiations they were also suspended in the water bath, shielded from all radiations. Between radiations they were placed in the seventh compartment of the large finger bowl.

The total amount of radiant energy to which the eggs, or tadpoles, were exposed daily was equalized for each group except the controls, which were not exposed at all. This equalization was accomplished by varying the time so that the value for IT was constant for all groups of eggs, where I was energy in terms of centigrade degrees and T was time in minutes.

DATA

The data in Table I represent the results of eight complete sets of radiation experiments for each period of 15 and 30 days, made over a period of five months. The eggs (or tadpoles) of a single experiment were at all times kept at the same temperature. The total amount of radiant energy under the different filters was exactly the same, namely 125.00 centigrade-minutes per day. The single variable was the spectral region which was limited in each case by filters.

The length of the tadpoles at the end of 15 days showed a maximum deviation of 0.64 per cent from the controls, and at the end of 30 days this deviation was 0.63 per cent, which is exceedingly close

TABLE I

GROWTH-RATE OF TADPOLES UNDER DIFFERENT PARTS OF THE SPECTRUM USING EQUAL RADIANT ENERGY

Filter	Spect. Trans.	Color	Rad. Energy Cent. Min./ Day	Length (mm.) (15 days)	Length (mm.) (30 days)
No. 774	.365–3.5μ	Sunlight	125.00	15.58	22.10
No. 554	.390– .490	Blue	125.00	15.52	22.00
No. 401	.480– .580	Green	125.00	15.50	22.14
No. 349	.550– .700	Yel. red	125.00	15.52	22.10
No. 243	.620– .700	Red	125.00	15.54	22.00
No. 254	1μ–3μ	Infra-red	125.00	15.60	22.12
Control	0	Darkness	00.00	15.50	22.00

and represents a figure well within the limits of observational error. Since a total of 120 tadpoles were measured for each spectral region the averages are significant at least to 0.1 mm.

DISCUSSION

The human retinal cells are sensitive to only a narrow band of radiations (4,000–8,000 A) and this fact has caused biologists to be so prejudiced in their experimental work with light that they have assumed that what cannot be seen does not exist. The literature abounds in references to the biological effects of luminous intensities where the quality (wave-length) and energy (heat) of radiations were entirely ignored. The spectrophotometer, photronic photoelectric cell, or the human eye are insensitive to the infra-red which has often accompanied what was described as monochromatic light. This extra radiant energy might alone explain many of the experimental results. In any radiation experiment two of the three factors, wave-length, luminous intensity, and radiant energy, must either be controlled or shown to be negligible. It is necessary here to distinguish briefly between these factors and then evaluate their relative importance in respect to the growth-rate of the frog tadpole.

Quality or wave-length.—The quality of radiation is measured in terms of wave-lengths. The only qualitative (physical) difference between an X- or gamma-ray, an ultra-violet, a visible, an infra-red,

or a Hertzian wave, is a matter of wave-length. The human or insect eye and the frog's skin are not similarly sensitive to the various qualities of radiation.

Water is relatively transparent to the solar ultra-violet and the visible wave-lengths, but there is a rapid decrease in transparency toward the longer (red) wave-lengths. For this reason water is often used to absorb the infra-red rays, erroneously called "heat rays."

TABLE II

SPECTRAL, LUMINOUS, AND ENERGY TRANSMISSIONS OF FILTERS

Filter	Spectral Range (A Units)	Color	Rel. Lumin. Trans. (Foot-Candles) (per cent)	Rel. Energy Trans. (Paired Thermom.) (per cent)
YUNG'S FILTERS				
Aq. Gentian violet...	4,200–5,000 6,600+	Violet	31	45.5
Alc. Lyons blue.....	4,200–5,800 7,000+	Blue	14	43.0
Aq. Nickel nitrate....	5,000–6,000	Green	10	4.4
Aq. K. chromate.....	5,200–7,600+	Yellow	78	39.4
Alc. Fuchsin (basic)..	6,400–7,600+	Red	30	41.0
CORNING GLASS FILTERS				
No. 243.....	6,200–7,000	Red	25	66.94
No. 254.....	1μ–3μ	Infra-red	∞	59.19
No. 349.....	5,500–7,000	Lantern yellow	52	61.92
No. 401.....	4,800–5,800	Sextant green	3	20.04
No. 554.....	3,900–4,900	H.R. Lantern blue	4	24.05
No. 774.....	3,650–3μ	Pyrex (sunlight)	95	91.65

Organisms radiated in water cannot therefore receive equivalent benefit from the longer wave-lengths available to land forms.

Yung (1878) found that various colors affected differentially the growth rate of tadpoles of both *R. temporaria* and *R. esculenta*. His results indicated a descending order of effectiveness as: violet > blue > yellow > white > dark > red > green. In a later paper (1881) Yung showed similar results with *Loligo*, *Salmo*, and *Lymnaea*. He concluded that darkness retards growth, red and green are negligible, and that mortality is greater under colored than under white light.

A spectrophotometric analysis of the liquid filters used by Yung

shows that only the green was monochromatic. The violet and the blue transmitted red and infra-red, and the yellow transmitted some green and red. From a purely qualitative point of view, therefore, Yung's conclusions are open to criticism.

In Table II there is given a complete analysis of the filters used by Yung and in the experiments reported here.

In Yung's experiments the filters included two thicknesses of glass which did not reduce luminous transmission but did reduce energy transmission by 14 per cent.

Luminous intensity.—Luminous intensities (measured in terms of candle-power) refer to "light," which means radiation within the limits of the visible spectrum. According to W. E. Forsythe of the Bureau of Standards (correspondence): "The term candle is used to designate a certain intensity of light, meaning by light, radiation properly weighted as it affects the eye. . . . . Whenever the term candle is used it presupposes, of course, the human eye. Any radiation that is outside of the region of wave-lengths that are capable of affecting the human eye would have no candle power, however intense it might be." The sensitivity of photometers is not a horizontal line through the spectrum but is a curve just as peculiar to itself as the spectral sensitivity curve of the human eye.

Luminous intensities therefore deal with sensations in the human eye and therefore do not represent intensities in the sense of energy content of radiation. Their biological significance must always be related to light-sensitive organs. In no way can luminous intensity, wave-length, and radiant energy be shown to be directly related.

Table II gives the relative luminous transmissions of all filters considered. It will be noted that quite naturally the infra-red filter transmitted no light. These measurements were all made with the photronic cell. Until later stages in the development of the tadpole there are no eyes and the embryo may be considered as a black body with little if any reflection of radiations from the body surface. Since luminous transmissions of the various filters used varied from 0 per cent to 95 per cent, and the experimental results did not show corresponding differences in growth rate, in fact showed no differences in growth rate, we must conclude that so far as the frog's egg and tadpole are concerned, luminous intensities have no significance.

Radiant energy.—Radiant energy refers to a physical character-

istic of all parts of the radiation spectrum, from the gamma rays to the Hertzian waves. Absolute measurements can be made over the major part of this spectrum by means of thermopile, bolometers, or the paired thermometers, and the values may be expressed in terms of ergs or gm. cals. per sq. cm. per minute. Any blackened radiometer is a non-selective absorption medium, registering radiant energy irrespective of the quality or luminous intensity.

Table II shows definitely that there is no correlation between the spectral, luminous, and energy transmissions of the various filters. It will be noted that the energy transmissions of Yung's filters correspond very remarkably with the relative growth-rate of his tadpoles under different filters. Violet and blue transmitted the greatest amount of radiant energy and green the least.

An attempt was made to reproduce exactly the physical conditions of Yung's experiments in order to determine the total amount of radiant energy received by an egg or a tadpole during a single day and during the period of a complete experiment. With calculations based upon clear, constant, mid-summer skylight, over a daily period of 10 hours, through the various filters used, and estimating an average depth of 3 cm. of aquarium water through which the light had to pass to reach the organisms, the maximum daily exposure of a tadpole under each of the filters (calculated in centigrade-minutes) was as follows:

Filter	C°-Min.
Violet	27.616
Blue	22.533
Green	2.205
Yellow	20.647
Red	21.485

These values represent, at the most, about $\frac{1}{5}$ the total radiation received by each tadpole in the experiments reported here. The relative growth-rate of Yung's tadpoles under these various filters corresponds very accurately with the relative amounts of total radiant energy calculated.

The contention of Yung, often quoted, that the frog's egg and tadpole are differentially susceptible to various parts of the spectrum cannot therefore be accepted for the following reasons: (*a*) the filters were not monochromatic, (*b*) there was no attempt to measure or control the heat factor, (*c*) the light source (skylight) was relative-

ly weak and quite variable, (*d*) the fact that the green plant food used for the tadpoles also had minimum growth rate in green light was not recognized. This last factor might very well have been one which caused the death of tadpoles kept under green light.

It has been the purpose of these experiments to equalize all factors except the quality of radiation. This one variable was controlled by appropriate filters. It was shown that luminous intensities are of no specific significance, apart from radiant energy, in respect to the rate of development of the frog's egg and tadpole.

CONCLUSIONS

1. Within the limits of the solar spectrum, the quality of radiation does not show any differential effect on the growth-rate of the frog's egg or tadpole if the radiant energy is equalized at the different spectral regions.

2. Explanations are offered for the results secured by Yung.

3. The biological significance of wave-length, luminous intensity, and radiant energy is discussed, in view of the data presented.

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THE INFLUENCE OF TEMPERATURE UPON THE RESPIRATORY METABOLISM OF THE PACIFIC KILLIFISH, FUNDULUS PARVIPINNIS

(Six figures)

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A LARGE number of experiments have been undertaken in an attempt to discover the relation between temperature and respiratory metabolism in cold-blooded vertebrates, but relatively few of them have been made under "standard conditions." Vernon's experiments (1895, 1896, 1897), which were quite complete, are contradictory in some cases. For example, he finds that *Rana temporaria*, in one series of experiments over a temperature range of 2°–30°, has a constant metabolism from 6° to 17.5° on warming, and more or less so between 17.5° and 12.5° on cooling. Again, there are many cases in which he finds that the metabolism remains nearly constant over considerable temperature intervals.

By far the most complete series of experiments on temperature relations in poikilotherm vertebrates are those of F. G. Benedict (1932). He utilized alligators, lizards, tortoises, and four genera of snakes. The greater part of his work was confined to the latter. He found from experiments on fourteen boas that the heat production per square meter of body surface is no more uniform than heat production per kilogram of body weight, while with pythons the metabolism per unit of weight is even less irregular than that per unit of body surface. In Figure 67 he has plotted his average values for the rates of metabolism of four species of snakes (gopher snakes, rattlesnakes, pythons, and boas), showing relation between temperature and body weight. It is quite clear from this graph that in no two species is the relation the same. No two curves are parallel. This is certainly proof that no single curve could represent the relation between temperature and standard metabolism in reptiles, much less in poikilotherms in general.

Some experiments have been conducted in an attempt to establish this relation in fishes, notably by Jolyet and Regnard (1876) (1877), Knauthe (1898), Montuori (1907, 1913), Lindstedt (1914), Ege and Krogh (1915–16), and Gardner and King (1922). Cronheim (1911) has analyzed the results obtained by Knauthe. They show an irregular rise in the respiratory exchange of carp, with temperatures ranging from 7° to 25°. The values obtained in the summer were somewhat higher than those obtained in the winter. Later experiments made by Lindstedt gave somewhat more regular results. Gardner and King (1922) found that the oxygen absorbed by small brown trout appeared, within limits, to be proportional to the temperature, and was approximately doubled for a 10° rise in temperature. Results with large fishes (70–150 grams) were very variable. Their results suffer from the inaccuracy of the method.

The experiments of Ege and Krogh (1915–16) on a normal and narcotized goldfish are the most reliable, but since these experiments were performed with but one individual, and that a fresh-water fish, it was desirable to know whether the same relations would hold with marine fishes, particularly if several fishes of approximately the same weight were used. Would the relations for a narcotized goldfish be the same as for several fishes of a species when placed together in a container which allowed them a certain freedom of movement? Individual variation in the rate of metabolism of fishes of equal size and, in some cases, a higher rate of metabolism in a large fish than in a small one (Table I), made it seem reasonable to expect that if several fishes of approximately the same size were used in a single container, the results would give a more accurate picture of what is actually taking place. At least, individual differences in metabolic rate would cancel one another and the major fluctuations that occur from hour to hour when only one fish is used should be smoothed out. Furthermore, it was desirable to know whether or not several fishes in a container which provided them some freedom of movement would show a greater increase in oxygen consumption with increased temperature than Ege and Krogh found for a single goldfish. Since his values for the narcotized and normal fish were nearly equal, it is evident that the normal fish remained quiet throughout the experiment.

Before proceeding with the experiments on the effect of temperature on the rate of metabolism, it was necessary to know what factors should be considered that might invalidate conclusions drawn from the experimental data. The relation between oxygen tension and oxygen consumption; the effect of increased carbon dioxide, of light, of the size of the containers, and of numbers of individuals in the containers upon oxygen consumption, were the considerations of most importance.

The independence of most fishes upon the oxygen concentration of the water, down to 1 cc. or less per liter, has been established by several investigators. Winterstein (1908) concluded that with *Leuciscus erythrophthalmus*, the oxygen consumption within wide limits was independent of the oxygen tension. Henze (1910), from the results of his experiments on *Coeris* and *Sargus annularis*, draws the same conclusion.

In a very exact experiment under standard conditions, Gaarder (1918) corroborated the findings of Winterstein and Henze. Gaarder's experiments were conducted with *Cyprinus carpio*, and the oxygen concentration varied from 1 to 16 cc. per liter. Over that range, as may be seen from his graph (p. 113), there was a gradual increase from the lowest to the highest tension, but over the range used in my experiments (from 3.5 to 5.6 cc. per liter), the oxygen consumption is virtually independent of the tension. Amberson, Mayerson, and Scott (1924) found that when the oxygen tension diminished slowly, *Fundulus heteroclitus* was able to remove equal quantities of oxygen in equal times down to about 10 per cent of oxygen saturation, showing that the oxygen consumption in this species is independent of oxygen tension over a wide range. They found, however, a slight dependence when the oxygen tension was reduced rapidly. Toryu (1927) using *Carassius auratus*, found that the oxygen consumption was independent of the oxygen tension of the water until its partial pressure was reduced to about a tenth of the normal. This is in line with the work of Crozier and Stier (1925*c*) on opercular breathing rhythm of the goldfish. These investigators found that "comparative tests in which the oxygen concentration was varied between 0.68 and 6.2 cc. per liter showed no significant effect of oxygen concentration upon breathing frequency."

The opposite view, that the oxygen consumption of some fishes, at least, is influenced by oxygen tension, is upheld by Powers and Shipe (1928) and by Hall (1929). The former were concerned with the effect of oxygen tension during the first few minutes after the fish were placed in the respiratory chambers. However, as Keys (1930*b*) has pointed out, the data given leave much to be desired. The authors do not consider the effect of temperature, which varied from 12° to 20° throughout their experiments. Temperature, as I shall show, is a factor of extreme importance in any comparative studies on respiration of fishes. Furthermore, these authors do not differentiate between the effect which may be due to carbon dioxide and that due to low oxygen supply. Powers (1932) objects to Keys' criticism, saying that Keys missed the point of his 1928 paper, but Powers still does not refute the claim that his data do not warrant the conclusions he draws. Hall (1929), working with three species of fishes, finds a differential relation in their ability to extract oxygen at varying tensions. The scup, a very active fish, is independent of the oxygen tension between 40 mm. and 120 mm. of mercury. The oxygen consumption of the toad fish, on the other hand, was directly proportional to the oxygen tension over the whole experimental range, while the puffer occupied a position intermediate between the two. He states that the toadfish can readily survive 24 hours in oxygen-free water. This is rather difficult to believe. Here, again, the method is questionable, since Hall allowed only an hour to elapse from the time of transfer to the respiratory chambers before he made his first oxygen determinations, a source of error to which I have called attention (Wells, 1932). A further source of error lies in his method for controlling the oxygen concentration of the incoming water. It has been pointed out by several workers, M. M. Wells (1913, 1918), Shelford and Powers (1915), and Powers (1921, 1922), that fishes are very sensitive to small changes in the pH or in the carbon dioxide of the water, and, although Powers' results have been questioned by Pruthi (1927), it is imperative in studying the effect of one factor to eliminate as far as possible other factors which may tend to invalidate the results.

Although I have performed no specific experiments for the purpose of studying the relation of oxygen tension to oxygen consumption,

analysis of a vast number of data gathered over a period of 4 years gives no indication that a positive relation exists between the two factors. As this corroborates Keys' findings in this laboratory with the same species of fishes, *Fundulus parvipinnis*, it seems justifiable to assume that, within the range of variation with which my experiments are concerned, the metabolism of these fishes is independent of the oxygen tension.

Concerning the possible effect of hydrogen ion concentration on the metabolism of marine fishes, there are few data available, and none on *Fundulus parvipinnis*. Powers' (1922) paper, already quoted, deals with five species of marine fishes, among them the Pacific herring, *Clupea pallasii*, which seemed to be the most sensitive to change in pH. He states of these fishes that "The lowest tension at which the herring, *Clupea pallasii*, can extract oxygen from sea water is when the hydrogen ion concentration is ± 7.68 pH." He found these fishes most abundant in Puget Sound when the pH of the waters was about 7.73–7.78. Gutsell (1929) found that water containing 40 p.p.m. of carbon dioxide is not markedly harmful to brown trout and that they remain perfectly healthy in water containing 28 p.p.m. Keys (1931) calculates that if practically all of the water available to the fish passes through its gills, the pH of the inspired water would not be changed more than one or two hundredths of a pH unit. I have found on various occasions that when the fishes were forced to use over one-half of the available oxygen, the pH of the inspired water was not changed more than one-twentieth of a pH unit (8.10–8.05). It is obvious from these data that when no more than one-third of the oxygen available is extracted by the fishes, even though they rebreathe part of the water, there can be no measurable effect on their oxygen consumption due to increased hydrogen-ion concentration.

As far as the experiments discussed in this paper are concerned, the effect of light on the rate of metabolism can be neglected. In those experiments where observations were made at regular intervals over a period of 24 hours or longer, the fishes were subjected to subdued daylight during the daytime, and subdued lamplight at night. There is no indication of a diurnal rhythm, as I have shown elsewhere (Wells, 1932).

Schuett (1933) studied the effect of numbers of individuals on the rate of respiratory metabolism of fishes of four species, one of which was the Atlantic killifish, *Fundulus heteroclitus*. He finds that there is a consistent decrease in their rate of metabolism when he uses one, two, and four fishes in containers of the same size, but when the size of the containers used was in proportion to the number of fishes there was no difference. The reasons for this "group effect," he states, are not clear. Although he used a flowing water system which, he assumes, insured that reduced oxygen tension and increased carbon dioxide could not affect the metabolism, his experiments were of such short duration (10 hours) that his results were questionable. In a more recent publication Schuett (1934) states that the differences he found in his earlier investigations were due to error in the method of oxygen analysis.

In 1930 I performed one series of experiments with nineteen *Funduli* ranging in weight from 4.4 to 8.0 grams, in which the oxygen consumption of the fishes was first measured individually, and then as a single group. The entire experiment was completed in 11 days. There was no difference in their rates of metabolism under the two conditions.

I have shown in an earlier publication (Wells, 1932) that the determination of "normal" metabolism of *Fundulus* is not possible until the fishes have been undisturbed in the experimental environment for approximately 24 hours. In that paper I have defined normal metabolism and the conditions under which it may be obtained with *Fundulus*.

I wish to call attention now to the nutritional state of the fishes during the experimental period. I carried out several experiments in 1930 to determine the effect of lapse of time from feeding on the rate of respiratory metabolism of fishes. I chose *Girella nigricans* for this work because this is a very active fish, and should show effects of starvation more quickly than less active species. Several individuals were used. The values of the oxygen consumption of six of them, expressed in cubic centimeters per hour per gram of fish, are tabulated in Table I. The animals were first placed in the respiratory chambers 24 hours after they had taken food, and 4 hours after transfer to the chambers two values of the oxygen consumption were obtained.

They were then replaced in the aquarium. The succeeding periods after feeding were 48 hours, 72 hours, 96 hours, 168 hours, and, in two cases, 192 hours. In each case the values for the oxygen consumption were obtained at the same intervals after introduction into the chambers. With the exception of fish G14, the values are all of the same order of magnitude, and there is no significant change in the oxygen metabolism during a period of 7 days after feeding. The high

TABLE I

THE O_2 CONSUMPTION OF *Girella* AS AFFECTED BY THE INTERVAL AFTER FEEDING
THESE VALUES ARE TWO SUCCESSIVE HOURLY DETERMINATIONS
COMMENCING 4 HOURS FROM TIME OF INTRODUCTION
INTO RESPIRATORY CHAMBERS

(O_2 consumption in cc./hr./gram of fish, temperature 20° C.)

Hrs. from Feeding	Wt.	O_2 Consumption	Wt.	O_2 Consumption	Wt.	O_2 Consumption	Wt.	O_2 Consumption	Wt.	O_2 Consumption	Wt.	O_2 Consumption
24.....	8.5	.153 .161	10.2	.136 .134	2.2	.23 .24	3.0	.21 .21	4.7	.23 .20	5.7	.23 .24
48.....	8.0	.150 .130	10.0	.204 .170	2.2	.23 .23	3.0	.21 .20	4.5	.20 .20	5.4	.18 .18
72.....	8.0	.134 .130 .131	10.0	.250 .236 .242	2.2	.24 .24	3.0	.22 .21	4.5	.20 .19	5.4	.18 .17
96.....	8.0	.147 .126	10.0	.20 .18	2.2	lost .24	3.0	.21 .20	....	.....	.....	.....
168.....	8.0	.140 .130	10.0	.19 .18	.....	.....	.....	.....	4.3	.20 .20	5.2	.23 .21
192.....	8.0	.138 .150	10.0	.167 .170	.....	.....	.....	.....	.....	.....	.....	.....

values for G14 after the first day may have been due to abnormality of the fish, or merely to the fact that the fish happened to be more active during the 2 hours in which the water samples were collected. Similar exceptionally high values may occur in any serial determinations of the oxygen metabolism of fishes. The results of these experiments are in accord with the findings of Lipschutz (1911), and Dakin and Dakin (1925). The latter authors found that the oxygen consumption of well-fed fishes varied considerably and was lower after

the fishes had been deprived of food for 48 hours. Whether the amount of oxygen consumed in the first 7 days of starvation represents the normal requirement in nature, it seems justifiable to assume that values obtained during the period from the second to the seventh day of starvation are comparable and represent normal metabolism under laboratory conditions. Finally, in all cases where the results from two or more sets of data were to be compared, the interval after the time of feeding was the same, a fact which eliminates any variation due to differences in the nutritional state.

MATERIALS AND METHODS

The criteria of an efficient and accurate method of measuring the respiratory exchange of aquatic animals are (1) that the physical and mechanical conditions remain constant, (2) that duplicate samples of water drawn from the collecting cylinders check, and (3) that the rate of flow of the water passing through the respiratory chambers be maintained invariable over long periods of time. It is well known that in a normal animal the respiratory exchange may vary 100 per cent and more (Krogh, 1916), and that the physiological condition of the animals varies from day to day, even from hour to hour. It is the rule and not the exception that successive hourly values of the oxygen consumption may vary considerably even when no visible movements of the animal are discernible. Duplication of results with the same animals on two different occasions is not a measure of the efficiency of the apparatus, nor is failure to duplicate results a reflection upon the method. By the method used in my investigations the physical conditions of the medium, such as temperature, quantity of dissolved oxygen, etc., can be altered at desired intervals without any mechanical disturbance of the animals, thus insuring that for any given period and for as long as is desired the environment will remain constant.

The apparatus employed in these investigations has undergone considerable modification since the first set-up was assembled in 1929. When it was found necessary to continue single experiments over periods of several days, still further changes were required in order to insure that a given experiment could be carried to completion with a minimum chance for accidents. The only similarity be-

tween the final model and the first one which I used is the basic principle: that of a continuous stream of water passing through the chambers in which the fishes are imprisoned, to insure that they receive a sufficient quantity of respirable oxygen. This principle has been used in various modifications first by Gréhant (1886), subsequently by Vernon (1896), Winterstein (1908), Ege and Krogh (1915–16), Gaarder (1918), Keys (1930*a*),[1] Wells (1932), and others.

In the apparatus (Fig. 1) used in these experiments the sea water is led from the supply line through a filtering chamber which prevents detritus and macroplankton, such as copepods, from reaching the supply jars. The filtering chamber is a 35 mm. glass tube filled with absorbent cotton which can be replaced when it becomes fouled. The jars are maintained at a constant level by means of an overflow tube, assuring that the pressure of water in the respiratory chambers will remain constant. The water passes from the supply jars, at a pressure of 150 centimeters, through a block tin pipe and coil into the respiratory and control chambers, which are immersed in a constant temperature bath, the temperature of which is controlled to within one-twentieth of a degree Centigrade. The water passing through the coil immersed in the constant temperature bath is brought to the temperature of the bath before reaching the respiratory chambers. After leaving a respiratory chamber the water passes through a settling flask and then through a valve which regulates the rate of flow. These settling flasks deserve some explanation because they make possible the removal from the respiratory chambers of fecal material which otherwise would pass through the regulating valves and might either change the rate of flow or stop it entirely. The use of these flasks makes it possible to flush out the chambers without disturbing the fishes or necessitating readjustment of the regulating valves. It also prevents any fecal material from passing into the collecting cylinders where it might interfere with the Winkler determinations of the dissolved oxygen (Henze, 1910). In the bottom of a 500 cc. distilling flask is blown a glass tube provided on

[1] My first model constructed in 1929 with the assistance of A. B. Keys was used in the study of amytal anesthesia in fishes (Keys and Wells, 1930). Keys later built an improved model and described it (Keys, 1931). The improved type of collecting cylinder designed by him was incorporated into my present apparatus.

the end with a short rubber tube closed with a pinchcock. The water from the respiratory chamber enters the neck of the flask and passes out the side tube to the valve. Any fecal matter settles at the bottom

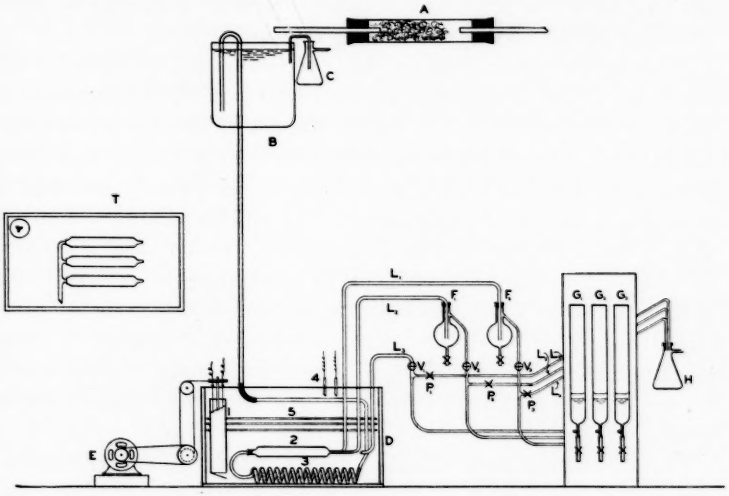

FIG. 1.—Diagram of respiratory apparatus used in determining O_2 consumption of fishes.

Sea water is led from the supply line through filtering chamber *A* into a reservoir *B* which is maintained at a constant level by means of the flask *C*. From the reservoir the water passes through a block tin coil (3) immersed in the constant temperature bath *D* to the respiratory chambers, only one of which (2) is shown in the cross-sectional diagram of the constant temperature bath. A top view (*T*) of the bath is shown with the three chambers in place. The water in the constant temperature bath is maintained at a constant temperature by means of the thermostat (4). The heating and stirring unit (1) and the cooling coils (5) are shown in cross-section. The stirring unit is driven by the motor *E*. The water passes from respiratory chambers through lines L_1 and L_2 into settling flasks F_1 and F_2, then through valves V_2 and V_3 either to collecting cylinders G_2 and G_3 or is by-passed to the sink through lines L_5 and L_6. The flask *H* is used to maintain a constant back pressure on the collecting cylinders. The pinchcocks P_1, P_2, and P_3 make it possible to shift the flow of water from the collecting cylinder to sink without changing the rate of flow. Water from the control chamber passes through line L_3 and valve V_1 either to collecting cylinder G_1 or to the sink.

of the flask and can be easily removed by releasing the pinchcock. The greatly increased rate of flow while the pinchcock is released serves to flush out the respiratory chamber without making it necessary to readjust the regulating valve after the pinchcock is replaced.

The rate of flow of the water is regulated with a screw clamp on the rubber tubing which leads from the settling flask to the collecting cylinder. Between the collecting cylinder and the regulating valve is a by-pass line which makes it possible to remove samples of water from the cylinder without changing the rate of flow.

With the exception of a few *Girella nigricans* used to determine the effect of feeding on oxygen consumption all of the fishes employed in these experiments were the Pacific killifish, *Fundulus parvipinnis*. These fishes are available most of the year in False Bay, a small body of sea water near San Diego, into which the San Diego River empties at intervals during the rainy season. The salinity of the bay water is usually quite similar to that of the ocean (Moberg, 1927). These fishes are extremely hardy and can be handled freely, even kept out of water for several minutes at a time without injury to them. Keys (1931) has shown that they may be transferred directly to fresh water without necessary damage. I have kept some of them in the sea-water supply of the laboratory for over two years in perfect health.

The oxygen was determined by the Winkler method, which is so generally in use that it does not require discussion. It should be emphasized, however, that the amount of nitrite present in the sea water, even when the fishes are forced to use one-half of the available oxygen, is so small that no modification of the Winkler method is necessary. The addition to the sea water of 0.03 mg. of nitrite expressed as nitrogen had no effect on the end-point in the Winkler titration. It was found that under the most unfavorable experimental condition the greatest amount of nitrite present was 0.004 mg./L. (expressed as nitrogen).[2]

EXPERIMENTAL RESULTS

In December of 1930, twenty-four fishes[3] were selected, each of which weighed approximately 4.25 grams; their combined weight was 102 grams. A rigid feeding schedule was followed so that in all

[2] These data should dispose of such criticisms of the Winkler method, as those of Schuett (1934), in so far as respiratory work with marine fishes is concerned, when a flowing water system is used.

[3] Of these 24 fishes, 7 died before the experiment was more than a third finished. Their death was due to the disease caused by *Achromobacter ichthydermis* (Wells and ZoBell, 1934).

cases the fishes were in the same nutritional state. In this experiment the values for the oxygen consumption were obtained after the fishes had been in the experimental environment 5 hours, and each point on the curve (Fig. 2) represents an average of four hourly determinations over a period of 4 hours. These averaged values are represented by solid black circles.

As the metabolism at some of the duplicated temperatures in this series showed considerable variation, it was thought advisable to repeat the experiment with another group of fishes. The next set, Experiment 2, included twenty-three fishes whose average weight was approximately 5.3 grams. This weight increased slightly as the fishes were used during the months of April and May, which I shall show constitutes a period of high metabolism, regardless of the temperature. These values, which were obtained under the same conditions as those of the first series, are plotted in Figure 2, and represented by horizontal half-black circles. The same variation occurred in this group as had occurred in the first, and at that time I was unable to account for it. I have taken the liberty to plot on this graph the values obtained by Ege and Krogh (1915–16) for a single goldfish. Their values have been reduced from kilogram and minute to grams per hour.

In the light of more recent experiments which were designed to study the effect of continued constant temperatures and season on the rate of metabolism, and which will be discussed later, these variations, I am convinced, can be logically explained.[4] There is good evidence that a seasonal variation occurs in the normal metabolism of these fishes, not only in relation to the temperature to which the fishes are subjected, but entirely independent of it (Wells, 1935). The lowest level seems to occur during the months from August to December, and the highest from January to March. In Figure 2, series *a* (solid circles), it will be noted that the values for the respiratory metabolism obtained for any given temperature in February and March are, in every case, higher than those obtained in December and January. In series *b* (half-black circles), it can also be seen that the May values are higher at 8° and 13° than the ones obtained in

[4] The values obtained in these experiments are subject to the same criticism as the one discussed on p. 199.

April. This may be due in part, however, to the onset of the breeding season. It may be concluded, therefore, that in any experiments designed to study the relation of temperature to metabolism in fishes, seasonal variations must be considered.

In view of the controversy which I shall discuss later over the application of mathematical formulas to data derived from experiments

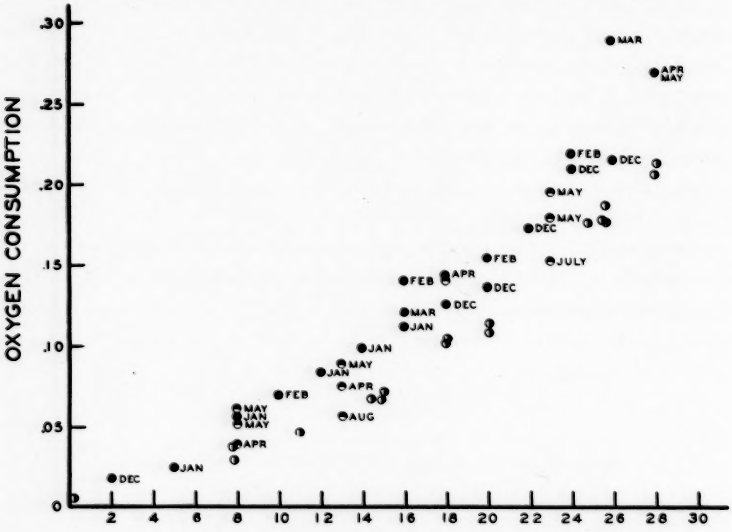

FIG. 2.—Relation of oxygen consumption to temperature.

Each point represented by solid and horizontal half-black circles on the graph is the average of four hourly values obtained after the fishes had been 5 hours in the respiratory chamber, and represents oxygen consumed per hour per gram of fish. The solid circles are the values for the first experiment, horizontal half-black circles for the second experiment. The vertical half-black circles represent values obtained by Ege and Krogh (1915–16) for a single goldfish.

on the effect of temperature on the rate of biological processes and in view of my findings (Wells, 1932) that the oxygen consumption of *Fundulus* in the post-24-hour period is lower and more constant, it seemed highly advisable to attack the problem from another angle—that of the relation of temperature to metabolism when successive one-degree increases of temperature are employed without disturbing the fishes.

TABLE II

A COMPARISON OF THE OXYGEN CONSUMPTION OF LARGE AND SMALL FISHES IN THE CONTINUOUS-TEMPERATURE EXPERIMENTS 3, 4, AND 5*

Temperature °C.	Experiment 3		Experiment 4		Experiment 5	
	O_2 Consumption, cc./hr./gr.		O_2 Consumption, cc./hr./gr.		O_2 Consumption, cc./hr./gr.	
	Large Fishes	Small Fishes	Large Fishes	Small Fishes	Large Fishes	Small Fishes
10	.....	.....	.047	.067	.055	.066
	.....	.....	.079	.066	.045	.065
	.....	.....	.052	.074	.062	.065
	.....	.....	.051	.063	.047	.061
11	.....	.....	.069	.079	.....	.....
	.....	.....	.050	.067	.....	.....
	.....	.....	.045	.063	.....	.....
	.....	.....	.075	.082	.....	.....
12	.097	.113	.069	.086	.058	.075
	.090	.106	.083	.103	.056	.075
	.088	.099	.077	.083	.070	.111
	.079	.096	.053	.077	.063	.092
13	.060	.124	.094	.089	.....	.....
	.065	.131	.096	.087	.....	.....
	.061	.136	.087	.092	.....	.....
	.098	.136	.060	.088	.....	.....
14	.090	.153	.116	.092	.091	.158
	.072	.145	.105	.120	.101	.148
	.082	.143	.068	.102	.091	.142
	.063	.139	.065	.099	.072	.124
15	.092	.151	.092	.131	.....	.....
	.083	.145	.078	.108	.....	.....
	.092	.130	.105	.117	.....	.....
	.084	.155	.079	.105	.....	.....
16	.069	.188	.084	.110	.090	.134
	.088	.210	.111	.118	.117	.146
	.090	.220	.102	.117	.103	.134
	.091	.204	.085	.114	.093	.127
17	.099	.200	.086	.127	.....	.....
	.107	.178	.096	.127	.....	.....
	.102	.191	.133	.145	.....	.....
	.096	.181	.092	.124	.....	.....

* *Expt. 3.* Consecutive hourly values of the O_2 consumption of 13 fishes, total weight 82.4 gms., average 634 gms.; and of 26 fishes, total weight 57.5 gms., average 2.2 gms. Over a temperature range of 12°–22° C.

Expt. 4. Consecutive hourly values of the O_2 consumption of 13 fishes, total weight 85.2 gms., average 6.55 gms.; and of 23 fishes total weight 55 gms., average 2.4 gms. Over a temperature range of 10°–24° C.

Expt. 5. Consecutive hourly values of the O_2 consumption of 10 fishes, total weight 65.7 gms., average 6.57 gms.; and of 24 fishes, total weight 56.3 gms., average 2.34 gms. Over a temperature range of 10°–24° C.

TABLE II—*Continued*

Temperature ° C.	Experiment 3		Experiment 4		Experiment 5	
	O_2 Consumption, cc./hr./gr.		O_2 Consumption, cc./hr./gr.		O_2 Consumption, cc./hr./gr.	
	Large Fishes	Small Fishes	Large Fishes	Small Fishes	Large Fishes	Small Fishes
18	.110	.214	.108	.152	.099	.141
	.110	.200	.160	.218	.123	.230
	.109	.207	.139	.246	.131	.202
	.102	.210	.168	.260	.127	.197
19	.116	.273	.186	.240	.....	.....
	.126	.285	.186	.236	.....	.....
	.110	.280	.157	.248	.....	.....
	.113	.240	.155	.214	.....	.....
20	.123	.246	.171	.228	.175	.230
	.126	.314	.141	.263	.186	.250
	.115	.320	.168	.288	.162	.244
	.119	.313	.137	.280	.155	.230
21	.131	.280	.144	.282	.....	.....
	.154	.280	.173	.305	.....	.....
	.135	.268	.170	.246	.....	.....
	.139	.310	.161	.300	.....	.....
22	.168	.362	.154	.260	.176	.238
	.151	.376	.154	.242	.174	.240
	.176	.405	.183	.300	.168	.236
	.154	.360	.139	.294	.182	.230
23	.....	.....	.200	.204	.....	.....
	.....	.....	.145	.326	.....	.....
	.....	.....	.210	.440	.....	.....
	.....	.....	.195	.372	.....	.....
24	.....	.....	.248	.370	.186	.254
	.....	.....	.212	.400	.202	.230
	.....	.....	.275	.410	.196	.250
	.....	.....	.195	.380	.226	.256

Three experiments were undertaken, to be designated 3, 4, and 5 (Table II). In the first, two groups of fishes, one averaging 2.2 grams, the other 6.34 grams, were placed in separate respiratory chambers at a temperature of 12° C. After 24 hours, four consecutive samples of water were analyzed over a period of 4 hours, then the temperature was raised one degree. This one-degree increase in each case required

about 15 minutes. After a period of 4 hours had elapsed, four more determinations were obtained, and the temperature again raised. This procedure was followed up to and including 22° C. Thirty days later another experiment was performed, using the same fishes (Expt. 4). For 10 days prior to this experiment the fishes had been kept at a temperature of 10°–12°. This time the starting temperature was 10° and the fishes were kept only 4 hours at each degree, instead of 8 as in Experiment 3, observations being taken hourly up to and including 24° C. For the fifth experiment the same two groups of fishes were used after an interval of nearly two months, during part of which time the fishes were kept at a temperature from 10°–12°, although they had to be kept for many days at room temperature because of difficulty with the sea-water supply. In this experiment the temperature increments were two degrees, over the range of 10° to 24°, and, as in the first series, four serial observations were made after the fishes had been subjected to each temperature for a period of four hours.

On the graph (Fig. 3) have been plotted the averages of the four values secured at each temperature. The three upper lines connect the values for the small fishes and the three lower lines connect those for the large fishes. The variations in the rate of metabolism for both groups of fishes will be discussed later. But first I shall compare the average values of the three "continuous" experiments on the large fishes with the average values of the first two experiments described (Fig. 2). These are both plotted in Figure 5. The dotted circles are the average values from the first two experiments (Fig. 2), and the solid circles, the averages of all the values obtained at each temperature for the large fishes in the three "continuous" experiments. While it is evident from an inspection of this graph (Fig. 5) that the general trend of the two curves is the same, closer inspection of the individual points will show that there is a marked difference between the two in the region between 14° and 19°. In the last two "continuous" experiments (4 and 5), there were sudden increases in the rates of metabolism in the region between 17° and 19° (Fig. 3), which in the averaged values (Fig. 5) appear at 18° and 19°. There was a slight increase at 16° in Experiment 3, but it may not be sig-

nificant. The values secured in the fourth experiment are plotted in Figure 4. In this figure, the original data, which are presented in table II (Expt. 4), have been smoothed by moving averages of five. In this experiment (Exp. 4), the temperature intervals of one degree

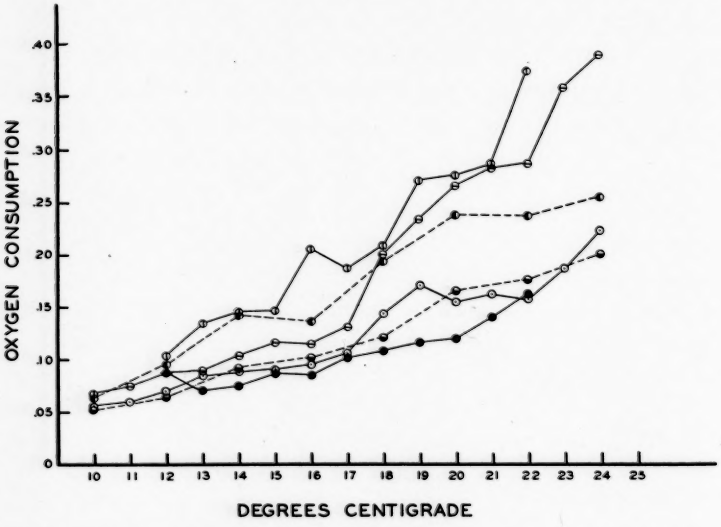

FIG. 3.—Relation of oxygen consumption to temperature.

Ordinates represent oxygen consumed in cc. per gram of fish per hour. Abscissas represent degrees C. Each point on the curve represents the mean of four hourly values of the oxygen consumption. The three upper curves show the values obtained for the small fishes: Experiment 3, vertical barred circles; 4, horizontal barred circles; 5, vertical half-black circles. The three lower curves are the values for the large fishes; Experiment 3, solid circles; 4, dotted circles; 5, horizontal half-black circles. Mean weights of the small fishes in the three experiments were respectively 2.2 gms., 2.4 gms., and 2.34 gms. Mean weights of the large fishes in the three experiments were respectively 6.35 gms., 6.55 gms., and 6.57 gms.

lasted 4 hours, and observations were made each hour for 60 consecutive hours. In this experiment and in the fifth (see Fig. 3), where the temperature increments were 2 degrees, the successive hourly values are no more irregular than in the third where the fishes were allowed 4 hours for adjustment to each degree rise in temperature before any observations were made. The hourly values for the third

and fifth experiments are given in Table II (Expts. 3 and 5). The fishes in the fourth and fifth experiments show higher metabolism at 18°, 19°, and 20° than they did in the third experiment, which may be due to the fact that they were subjected to cold water before the experiments were begun. In the third experiment there is a slight in-

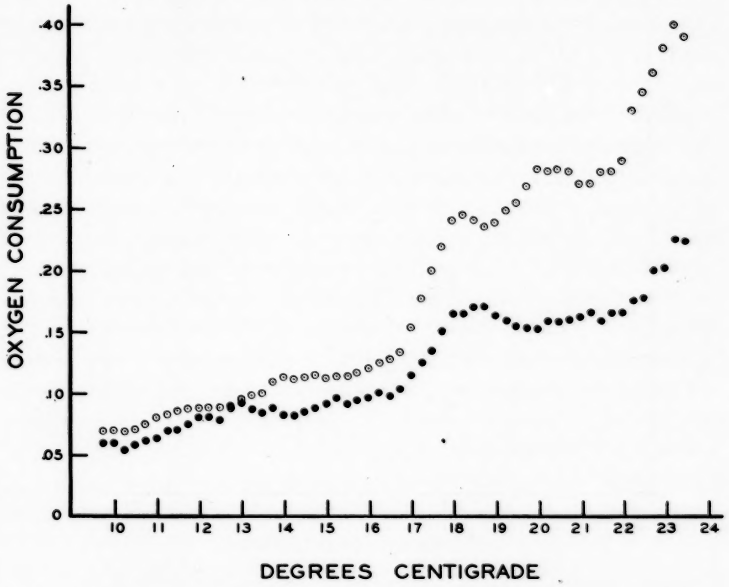

FIG. 4.—Relation of oxygen consumption to temperature.

Ordinates represent oxygen consumed in cc. per gram of fish per hour. Abscissas represent degrees C. The points on these two curves are the consecutive hourly values of the oxygen consumption smoothed by moving averages of 5. Dotted circles, small fishes (mean weight of 23 fishes is 2.38 gms.); solid circles, large fishes (mean weight of 13 fishes is 6.55 gms.).

crease at 16°, while in the fourth it takes place at 18° and is far more pronounced.

Although the general trend of the oxygen metabolism of the larger fishes coincides with that of the goldfish as found by Ege and Krogh (1915–16), the smaller fishes present an entirely different picture. The abrupt increases are not only more numerous but are more pro-

nounced. In the third experiment (vertical barred circle, Fig. 3) there are three apparent levels, with breaks at 13°, 16°, 19°, and 22°, while in the fourth experiment (horizontal barred circles, Fig. 3) only two sharp increases occur at 18° and 23°. In the fifth experiment (vertical half-black circles, Fig. 3) results are more difficult to inter-

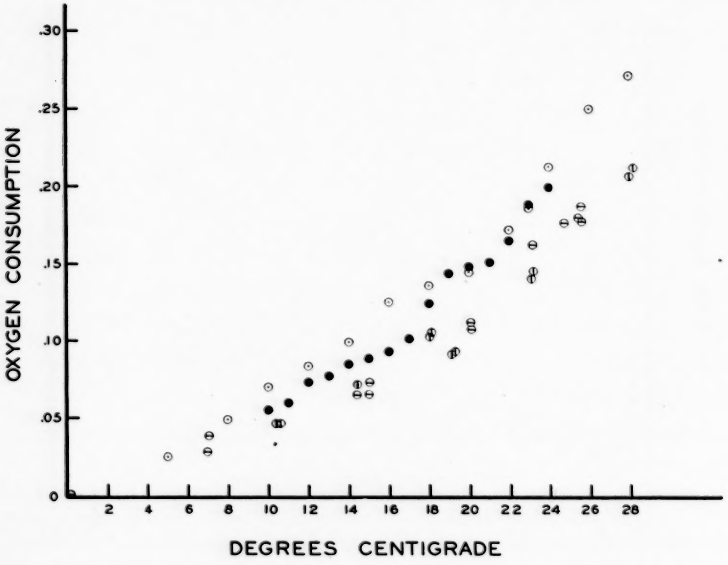

FIG. 5.—Relation of oxygen consumption to temperature.

The dotted circles are the means of the values obtained at each temperature in the first two experiments. Solid circles represent the mean values of the oxygen consumption of the large fishes obtained at each temperature in Experiments 3, 4, and 5. Vertical barred circles are Krogh's values for a narcotized goldfish, horizontal barred circles for a normal goldfish, both having been reduced from kilograms and minute to grams per hour.

pret. In a sense they are not comparable with the first two, because the temperature increases were 2 degrees instead of one. While the trend of this curve is the same up to 20°, the values at 22° and 24° seem much too low. This may be explained by assuming either that the fishes had a lowered vitality, indicated by a slight loss in weight, or that another degree rise in temperature was necessary in order to

initiate the sharp increase in their rate of metabolism that occurred in the two previous experiments at 22° and 23°.

The important considerations, however, are first, the difference in the trend of the curves for the small and large fishes and, second, that when small temperature increases are employed over a relative short period of time definite increases in the rate of respiratory metabolism do occur and are much more pronounced with the small fishes than with the large ones. The very close agreement in the trend of the curve for the large fishes between my results and the results published by Ege and Krogh lends support to an assumption that the reaction of small fishes to changes in temperature is either physiologically different or of a greater order of magnitude since the physical and mechanical conditions in each case were identical. In the third experiment a much longer time was allowed for the temperature of the fishes to reach an equilibrium with that of the surrounding medium than was provided by Ege and Krogh. In the fourth experiment 1 hour was allowed, which was the maximum time in their experiments. The hourly variations in the rate of oxygen consumption of the small fishes at any given temperature was slightly less in the second series than in the first up to 17°. From 17° to 24° these relations are reversed (see Table II, Expt. 4). A comparison of the first and last of the four hourly values at each temperature shows that there is no tendency either toward increase or decrease of metabolic rate during this period. It seems, therefore, that the time interval at each degree was sufficient.

Obviously it is impossible to draw any warranted conclusions from the data presented here; first, because there is too much variation between the values of the three experiments (3, 4, and 5) and second, because only the two weight groups have been used. It is unfortunate that fishes of these particular weights were chosen, because the oxygen metabolism of fishes from 2 to 3 grams in weight at a constant temperature is quite variable, as I shall show elsewhere. Until it is possible to perform experiments over the temperature range from 10° to 26°, utilizing several sets of animals ranging in weight from 0.6 grams to 12 grams, it seems to me futile to attempt to express a mathematical relationship between temperature and normal metabolism in fishes. It is possible that the results of such a series of

experiments would establish a family of curves from which could be theoretically predicted the normal metabolism of a fish of given weight at any temperature within its normal range. But I shall show in another publication that at least two other factors must be considered: namely, season and previous history as regards the temperature of the water from which the animal is taken; also the duration of time that it has been subjected to that temperature. However, these data do indicate very strongly that any attempt to correct to a constant temperature values of the oxygen consumption obtained at various temperatures will probably lead to erroneous conclusions if the animals differ greatly in size.

One explanation suggests itself for the increasing steepness at higher temperatures of the curves for the small fishes. It may be that with increasing temperatures the small fishes become increasingly more active than do the mature fishes, although from observation I have been able to detect no difference in their movements at high temperatures. An alternative explanation is that there is a greater economy of muscular movement in the larger fishes so that, to accomplish the same amount of work, i.e., in moving from one place to another, they expend less energy.

The sudden increases at certain temperatures in the rate of metabolism of both the large and small fishes, as shown in Experiment 5, are best illustrated in Figure 4. They must be significant since they occur in both weight groups in all three "continuous" experiments, although they are much more pronounced for the large fishes in the second of these (Expt. 4), where the temperature intervals were only half as long as in the first (Expt. 3), and where the fishes had been subjected to the lower temperature for a few days previous to the experiment. In the experiments of Knauthe,[5] Linstedt,[6] and Ege and Krogh (1915–16), the temperature data were rather scattered and those of Knauthe and Linstedt were obtained over a period of several months. As far as I know, no experiments have been performed with fishes in which 1 degree temperature increments were used in one

[5] Knauthe's results have been plotted by Cronheim (1911) and may be found both in his paper and in the paper by Ege and Krogh (1915–16).

[6] Krogh (1916) has plotted Linstedt's values from the data given in the latter's paper (1914).

continuous experiment. It seems significant that, in all of the above data, as well as in my own experiments, the oxygen metabolism was most variable between 13° and 20°.

Although Krogh (1916) has plotted Linstedt's data as oxygen consumption per square meter of body surface the values are very irregular between 13° and 17°, so much so that one wonders whether it is justifiable to attempt to represent them by a smooth curve. Lindstedt (1914) has plotted his own values as cc. of oxygen per kilogram of weight and, by averaging the very erratic values between 13° and 16°, has represented the relation between temperature and metabolism as nearly a straight-line function from 0° to 19° with a slight increase in the slope from 19° to 25°. With the exception of the break in Knauthe's curve between 14° and 18°, the relation between temperature and metabolism, as Krogh (1916) has pointed out, is practically linear. In Krogh's graph (1914) the values for the narcotized fish (my Fig. 5, vertical barred circles) are irregular between 18° and 20°, although his observations were not made at gradually increasing temperatures but at approximately 18°, 14°, 10°, 23°, and 28°. It should be pointed out, also, that his values for the normal fish form a more regular curve than that for the narcotized animal, probably for the reason that with the former no values were obtained between 15° and 20°. I wish to point out that if one picks values from the graph, Figure 5, at no shorter temperature intervals than were used by Ege and Krogh (1915–16), one can construct a smooth curve almost exactly parallel to his curve (e.g., Fig. 5, solid circles, at 10°, 14°, 18°, 22°, and 24°, or the open circles at 8°, 12°, 14°, 18°, 24°, and 28°). It is obvious that had determinations been made only at these temperatures, no serious breaks in the curve would have been detected.

One must therefore conclude from these experiments cited and from my own work that some change occurs in the metabolism of the fishes which incites sudden increases in their oxygen consumption (between 16° and 19°, usually, in the case of the larger fishes, and with the small fishes at approximately 22°). These breaks, it should be noted, are more pronounced with both the large and small fishes when they have been kept at the low temperature for some time previous to the experiment.

I have alluded to the controversy concerning the application of mathematical formulas to biological processes. It is well to begin with the discussion of the rule of van't Hoff by Ege and Krogh (1915–16). According to this rule, the velocity of a process increases in geometrical progression when the temperature is increased in arithmetical progression. It may be expressed as follows: when the temperature rises by 10° C. the velocity of most chemical processes increases twice to three times. Hence, the relation $\frac{K_t+10^\circ}{K_t}=Q_{10}=$ 2 to 3, where K is the velocity constant at temperatures t and $t+10^\circ$, respectively, and the quotient Q_{10} a constant generally called the temperature coefficient of van't Hoff. Ege and Krogh have shown that the curve they obtained, expressing the relation of temperature to metabolism for the goldfish, cannot be expressed satisfactorily by the rule of van't Hoff, since Q_{10} shows a steady and considerable decrease from 0° to 28°. Nor, if they use an "average Q_{10}," will it express the experimental results at all satisfactorily. They conclude that, with regard to biological processes, discrepancies of the same order as those between their observations and the van't Hoff curves are the rule and not the exception. I have calculated the Q_{10} values for some of my data and find that from 5° to 10°, $Q_{10}=4$, and from 18° to 28°, $Q_{10}=2$, hence the van't Hoff equation will not express my experimental results.

As my data show, within certain narrow temperature ranges there is the appearance of abrupt changes in the temperature relation occurring in the oxygen uptake of fishes. As a means of dealing with the "critical points" which occur in the temperature relations of many biological phenomena (Crozier, 1924*a*) the equation of Arrhenius (1915) has been applied. As usually written, it has the form

$$\frac{K_2}{K_1}=e^{\frac{\mu}{R}\left(\frac{1}{T_1}-\frac{1}{T_2}\right)}.$$

K_1 and K_2 are the velocity constants at the respective temperatures and may be expressed as Q_{10} which represents, in this case, the increase in velocity for a 10° rise in temperature. R is the gas constant 1.9898 cal/°C. The ratio between the velocity constants is related

logarithmically on the napierian base e to the gas content, the absolute temperature, and the constant μ. The equation as used would be

$$\mu = \frac{2 \times 2.303 \log_{10} \frac{K_1}{K_2}}{\frac{T_2 - T_1}{T_1 T_2}}.$$

The value μ is independent of the temperature and of the velocity of the process. Crozier has called it the "temperature characteristic." This formula has been employed by Crozier (1929*a*, *b* 1926*a*, *b*) and his co-workers (Crozier and Federighi, 1924*a*, *b*, *c*, *d;* Crozier and Libby, 1925; Crozier and Stier, 1925*a*, *b*, *c*, *d*), Crozier and Narez (1931) and by Glaser (1924, 1925*a*, *b*), Cook (1926), Navez (1929, 1930, 1931), and many others. Crozier maintains that any breaks in a curve drawn to represent the data obtained from a given experiment represent changes in the master reaction occurring in a given temperature range, and that these breaks most frequently occur at 4.5°, 9°, 15°, 20°, 27°, and 30°. For oxidative processes, such as respiration, the most frequent break occurs at 15°, below which the value of μ is approximately 16,000 and above which it is about 8,000. The decreasing values of μ with increasing temperatures for biological processes have been emphasized by Bélehrádek (1930). Crozier and Stier (1925*c*) find that the opercular breathing of the gold fish has a temperature characteristic of 16,500 over a temperature range from 5° to 25° C, but after exposure to water at a temperature of 25° for 3 hours the temperature characteristic is 8,260 from 9° to 25° C. (Crozier and Stier, 1926*c*). This seems to be a rather considerable change to be effected by so short an exposure to the high temperature.

Objections to the use of the Arrhenius equation to express temperature relations in biological reactions have been raised by Bélehrádek (1930), Heilbrunn (1925), and Fulmer and Buchanan (1929), with rebuttals by Navez (1930) and Stier (1933). It is evident that some inconsistencies exist when it is used to express the same function in different organisms. For example, Navez (1930) finds three temperature characteristics (16,400, 8,200, and 35,000) over a range

of temperature from 12° to 25° for the breathing rhythm of the dogfish, *Mustelus canis*. Crozier and Stier (1925*c*) found a value of 16,500 over a greater range of temperature for this same function in the goldfish. They (1925*c*, p. 701) have plotted the data obtained by Krogh on the oxygen consumption of the goldfish and find it has the same temperature characteristics for this function as for the breathing rhythm (16,500). However, Crozier (1924*b*, p. 200) previously obtained the value of 16,100 from the same data. The discrepancy is probably an error in calculation. To emphasize the variations in μ values I again refer to Crozier's 1924*b* paper. He has calculated the values of μ for Krogh's (1916) "curve of standard metabolism" and finds the values 14,670, 17,800, and another which he does not give, but since it is at the lower end of the curve it is probably around 35,000. He states:

> The individual sets of measurements pertaining to different species were 'averaged' by Krogh. It can be shown that the 'critical temperatures' at which a shift occurs from the dominance of one thermal increment to another, does not remain exactly constant for the different species; when series of determinations are averaged this results in a distortion of the fitted curve.

I have studied quite carefully Krogh's two curves (Krogh, 1916, pp. 96, 99), the one for goldfish and the one for standard metabolism, and so far as I can see, they are identical. The values for the normal and narcotized fish fall on both curves in the same places, and for the standard metabolism curve, Krogh has not averaged the values for the different species. Why, then, the numerous values (14,670, 16,100, 16,500, and 17,800) for the same curve? Crozier's statement that "not very much weight can be put, then, upon the curve of standard metabolism," is certainly not justified. Since the values of μ for respiratory velocity are so numerous (8,160, 9,400, 11,080, 13,700, 14,670, 16,100–16,800, 17,800, 21,000, 24,000, and 35,000) it seems that more weight should be given to a curve plotted from the actual values determined in the experiment than to a "constant" that is so variable. One other point to which I wish to call attention is the tendency of the proponents of the Arrhenius equation to omit from their publications the original data and the exact conditions under which they were obtained. If one wishes to attempt an analysis of these data by any other method, it is necessary to use a table

of logarithms and a slide rule, an indirect procedure and one in which much of the accuracy of the original values would be sacrificed.

Despite the foregoing criticism of the use of the Arrhenius equation it was deemed advisable to plot some of the data from the tem-

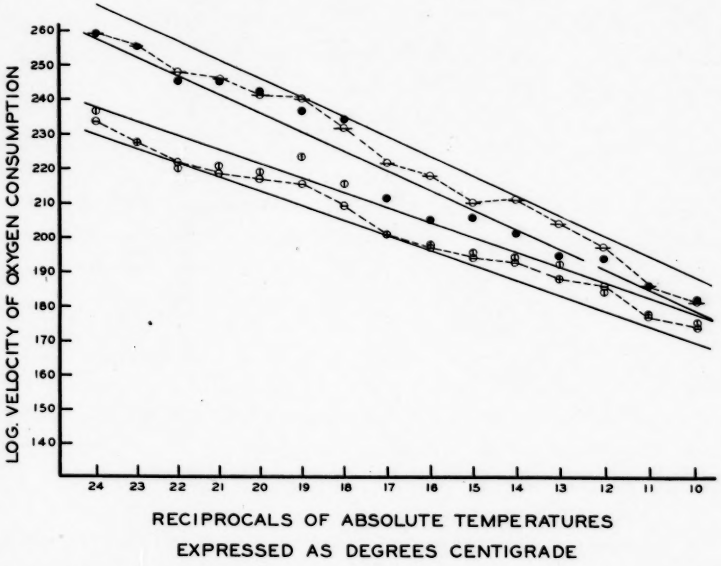

FIG. 6.—Relation of oxygen consumption to temperature.

Ordinates represent logarithms of the velocity of the oxygen consumption. Abscissae are the reciprocals of absolute temperatures expressed as degrees C. All the points on the curves are the means of the values at each temperature. Horizontally barred circles represent the large fishes in Experiments 3, 4, and 5. Vertically barred circles, large fishes in Experiment 4; barred circles upper band, small fishes in Experiments 3, 4, and 5; solid circles, small fishes in Experiment 4.

perature experiments and to find what values of μ would be obtained, and whether or not they were constant. Accordingly, the values of the second continuous experiment on both the small and large fishes and the average values of the three continuous experiments on both weight groups of fishes were so treated, and the data plotted in Fig. 6. It may be seen from this graph that the average values for the large

fishes of all three experiments (horizontally crossed circles) fall within two parallel bands, as do the average values for the small fishes (barred circles), although the band for the latter is slightly wider and the values of μ different—16,400 for the large fishes and 21,700 for the small ones. When the values for the second series, large fishes (vertically barred circles) and small fishes (solid circles), are considered, it is evident that several points do not fall within the bands, and that for the small fishes there are two discontinuous groups each with different values of μ. I wish to call attention also to the sinuosity of the points lying within the bands and to the high and low points in the two curves which fall at the same temperatures in each case. The high points for both curves fall at 24°, 19°, 14°, the lows at 22°, 17° and 11°, which indicate that the value of μ is constantly changing. To assign a constant value for the bands would be to obscure actual normal fluctuations in the respiratory metabolism. It must be concluded that the equation will not fit the data.

Obviously, one would not be justified in drawing conclusions as to the reasons for the sudden increase in the respiratory metabolism at certain temperatures until a sufficient number of experiments had been performed to determine the constancy and magnitude of the changes. It seems certain that they are not related to the size of the fishes except in degree of magnitude, but whether or not they will vary with season, or be shifted by subjecting the animals to high or low temperature for varying periods before introducing the temperature changes, must first be established before any theory as to the reasons for the changes can be stated. Analysis of my data (Fig. 3, Expt. 4, large fishes) indicate that an exposure to the low temperature for several days prior to the beginning of an experiment tends to emphasize the increased rate of metabolism at 17°–19°. These sudden increases may be due entirely to increased muscular activity and not be related to "standard metabolism" as defined by Krogh. Even if this were the explanation, it would not account for the abrupt changes in the muscular activity. Before the problem can be solved it will be necessary to utilize an experimental method that will enable one to separate "standard metabolism" from the increased metabolism due to muscular movements. This might be possible if one could employ a photoelectric method that would indicate at any

instant the concentration of oxygen in water passing through a suitable cell, and at the same time introduce an apparatus into the respiratory chamber that would measure the intensity of the muscular movements of the animals.

I can think of no better way to conclude this article than to quote from Barcroft's (1934) excellent book (p. 40) ". . . . it is clear that nature has learned so to exploit the biochemical situation as to escape from the tyranny of a simple application of the Arrhenius equation. She can manipulate living processes in such a way as to rule, and not be ruled by the obvious chemical situation. That is true at least over a wide range of temperature."

SUMMARY AND CONCLUSIONS

A brief discussion of the literature relative to the effects of temperature on metabolism of cold-blooded vertebrates is presented.

Factors that might invalidate conclusions, namely, variations in oxygen content and changes in pH, size of containers and numbers of fishes in the containers, are discussed. Literature is cited to show that under the conditions maintained in my experiments the variation in oxygen content of the water and changes in pH were so slight that they could be neglected in an evaluation of the data. Experiments are presented which indicate that size of containers and numbers of fishes in the containers had no noticeable effect on the rate of oxygen consumption of the fishes used in these experiments.

Experiments are presented to show that during the period from the second to the seventh day of starvation the oxygen consumption of the fishes remains very constant. Literature is cited to show that the results of these experiments are in agreement with the findings of other investigators.

The prerequisites of an efficient apparatus for measuring the respiratory metabolism of fishes are stated. The apparatus used in these investigations is described and improvements in it over those of the same type used by other investigators are emphasized.

From the results of experiments obtained on the relation of temperature to oxygen metabolism carried out under varying conditions and with fishes differing considerably in weight the following conclusions have been drawn:

1. The oxygen consumption of fishes of all sizes, of the species used (*Fundulus parvipinnis*), increases with increasing temperature but the increase is more pronounced with small fishes than with large ones. That is to say, the difference between the oxygen consumption by large and small fishes at a low temperature is much less than it is at a high temperature. These results make it evident that any attempt to correct to a constant temperature values of the metabolism of fishes of different sizes, secured over a wide temperature range, is not justified.

2. The relation between temperature and oxygen uptake of this species of fish cannot be satisfactorily expressed either by the rule of van't Hoff or by the Arrhenius equation.

3. The temperature of the water to which the fishes have been subject prior to the experiment, as well as the length of time of subjection to a given temperature, may influence considerably the temperature-metabolism relations at other temperatures.

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RELATIVE EFFECT OF BIOLOGICALLY CONDITIONED AND OTHER EXTREMELY HYPOTONIC WATER ON THE TISSUES OF THE MARINE TURBELLARIAN, PROCERODES WHEATLANDI[1]

(Eleven figures)

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IN A series of studies Allee (1928, 1929, 1933) has shown that the marine turbellarian *Procerodes wheatlandi* will survive longer in extremely hypotonic water if it has been biologically conditioned than if biological contamination is lacking, even though the total concentration of electrolytes is the same. The conditioning may be the result of a number of *Procerodes* living or dying and disintegrating in the hypotonic water, or it may result from *Euplanaria novangliae* (=*Planaria maculata*) or *Paramecia* living in the water. Also water extracts of *Procerodes* or of a marine gammarid, or of *Euplanaria novangliae* have the same effects. The protective value of the biologically conditioned water is maintained after dialyses to the same electrical conductivity as that of extremely dilute seawater controls. The protective material will stand boiling and will pass through filter paper.

When possible, such studies as differential survival should be supplemented by histological examination. At the joint suggestion of Professors Just and Allee, Hansborough (1931, 1934) undertook such an examination for *Procerodes* exposed to hypotonic sea water. Unfortunately, he was limited to the use of worms which had been shipped from Woods Hole to Washington, D.C. He also had difficulty with histological technique, so that only preliminary results were obtained. His work does serve to open the immediate problem for investigation. Previously, Lloyd (1914) had made histological studies on the related planarian, *P. ulvae* (=*Gunda ulvae*), to determine the influence of osmotic pressure on regeneration. More recently, Weil and Pantin (1931), while analyzing the adaptation of *P. ulvae* to

[1] I am deeply indebted to Dr. W. C. Allee for suggesting this problem, and for his continued interest and aid as the work has progressed.

salinity, reported that a brief examination had been made of sectioned worms for evidence of histological changes. These have been extended by Beadle (1934).

The *Procerodes* used in the present studies were taken from Allee's 1931 experiments, certain of the physiological results of which have been summarized elsewhere (Allee, 1933). Data are thus available concerning the experimental treatment of the worms sectioned and studied for the present report, and concerning their relative survival with respect to series of similar worms exposed simultaneously to various experimental conditions. An effort has been made to picture the typical histological changes which occurred in the above-mentioned experiments, and to contrast the changes in worms from hypotonic sea water with those from similarly hypotonic biologically conditioned media, and also to describe the sequence of histological disintegration, using all available material regardless of treatment.

TECHNIQUE

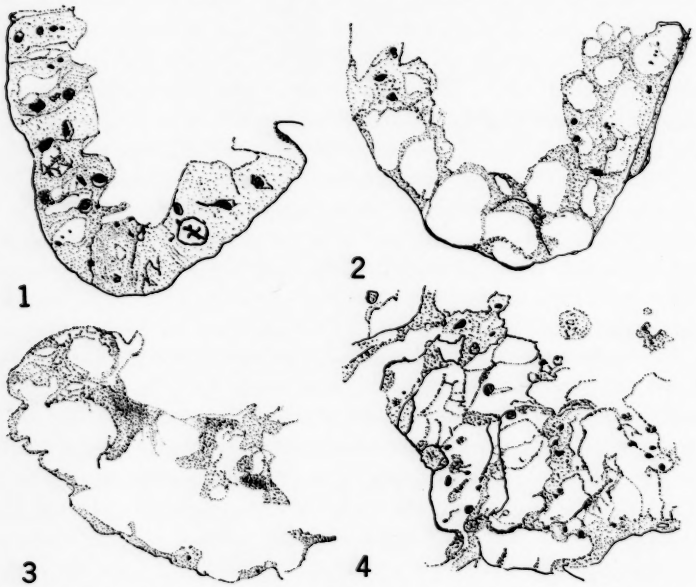
Thirty-four experimental and four normal worms were sectioned. When received for killing, each worm was already isolated in a watch glass with its experimental solution, into which it had been transferred from sea water after appropriate washings. After record had been made of its general condition, apparent swelling, and superficial lesions, if any, during the course of experimental treatment, the solution was carefully withdrawn by means of a pipette, and the worm quickly flooded with hot picro-formol (acetic). Care was taken to avoid injury to the worm in adding the fixative. The worm was allowed to remain in the fixative for 1 hour, was washed in 50 per cent alcohol, and was passed through the various concentrations of alcohol, alcohol and xylol, and xylol-paraffin, and through a series of paraffin changes in an oven held at 54° C., and finally imbedded in paraffin with a melting-point of 53° C. The worms were sectioned at 6 μ, stained in Heidenhain's iron haematoxylin, and counter-stained in eosin.

FINDINGS

A. HISTOLOGICAL CHANGES AND SEQUENCE OF TISSUE DISINTEGRATION

The study of histological changes and tissue disintegration was made from all available material, although a fairly complete record

could have been obtained from the series of six distilled water examples ranging in time of exposure from 1.16 hours to 2.50 hours. Except for time of appearance, the histological changes in worms in distilled water do not vary greatly from the changes in worms from the hypotonic sea-water solutions. A series of drawings has been



FIGS. 1–4.—Series of gut sections showing structural changes. (High power.)
FIG. 1.—Normal.
FIG. 2.—Early stage of cytolysis.
FIG. 3.—Late stage of cytolysis.
FIG. 4.—Stage showing rapid degeneration.

made to illustrate the progressive steps of the disintegrative process for each major tissue. Since the general sequence of events is similar in all, a rigid selection has been made for publication; and figures of the process of cytolysis in the gut and epidermis only are presented, together with a general low-power view from normal and treated worms.

The first tissue to show noticeable change is the gut. The cells fill

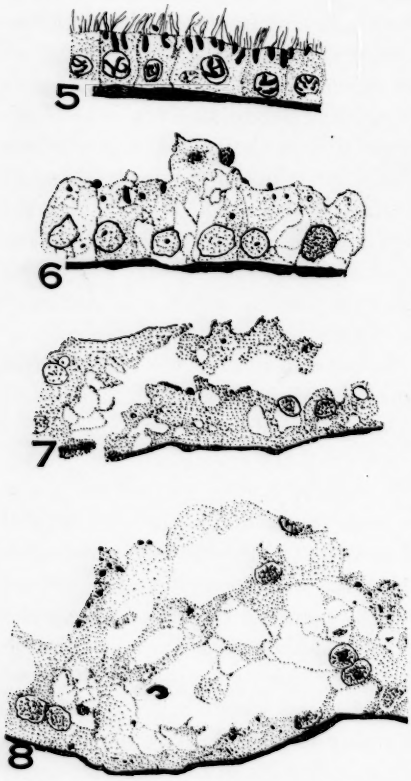
with large vacuoles (Fig. 2),[2] the food granules are either digested or egested, and the cell membranes break (Fig. 3). In cases where disintegration seemed to be taking place more rapidly, the gut cells pull loose from their basement membrane and fragment without losing all the food granules and becoming completely vacuolated (Fig. 4).

The muscle tissues are next affected. The first change noted is that of vacuolation accompanied by apparent swelling. The longitudinal layer shows these changes first in the ventral region where the layer is the thickest. The vacuoles which first appear are small and are scattered between the muscle fibers. These small vacuoles coalesce into larger ones, and the remaining protoplasm granulates. The changes in the circular layer of muscles are similar to those of the longitudinal layer. The oblique strands which run vertically through the body break as the other muscle layers cytolyze.

Following these changes in the gut and muscle, the reproductive organs and associated glands and ducts disintegrate. The collapse of the gut creates a cavity which may cause displacement and distortion of the reproductive structures and thus hasten the disintegrative processes. In the male reproductive organs, the mature sperm and the cells in the walls of the testis are the first to show cytological changes. The sperm become greatly twisted, as compared with their slightly curved and fairly even arrangement in the normal worm. Vacuoles appear around the nuclei of the cells of the testis wall, and the chromatin granules within the nuclei of the cells change character. The cytoplasm around the spermatocytes and the spermatids breaks away from the nuclei; and when the testis wall collapses, the sperm, nuclei, and remaining protoplasm are released into the body cavity, where they complete cytolysis. The vasa deferentia and sperm within them fragment and granulate without either swelling or vacuolation. The sheath surrounding the penis is often distorted, owing to the collapse of the gut or the extension of the proboscis, so that when the cells of the sheath become vacuolated they readily break apart. The penis becomes vacuolated in the outer muscular layer first, and then in the inner layers of tissue. The penis does not appear to increase in size, as do some of the other organs.

[2] All figures were drawn to scale with an A. C. Reichard camera lucida.

The genital atrium and the vagina, as well as the duct leading to the storage organ become vacuolated, collapse, and cytolize in the same manner as does the penis sheath.



FIGS. 5–8.—Series of epidermal cells showing histological changes. (Oil immersion.)

FIG. 5.—Normal.

FIG. 6.—Early stage of cytolysis.

FIG. 7.—Late stage of cytolysis.

FIG. 8.—Stage showing swollen and "foamy" appearance.

Of the female reproductive organs, the vitellaria, which in mature worms are large and full of yolk granules, are affected first. The yolk particles clump around the outer portion of the cell. As the gut disintegrates, the wall surrounding the grouped vitellarian cells breaks and the cells are released into the body cavity, where they undergo cytolysis. The shell gland which is located beneath the juncture of the oviducts breaks up in much the same manner as do the vitellaria. The eggs, located in a thin-walled ovary, are affected first by the formation of vacuoles in the outer cytoplasmic regions. Nuclei which contain chromosomes resist cytolysis longer than those with the chromatin in a fine spireme or resting state. The nuclear contents fragment, and the nuclear membrane breaks as the egg cytolyzes. The accessory female organs follow the usual method of cytolysis described above.

The epidermis, although one of the first tissues to show the effect of adverse treatment, does not completely collapse until many of the other tissues have completely cytolyzed. The first noticeable change is that of the appearance of small vacuoles around the nuclei of the cells, which increase in size and merge to form large vacuoles (Fig. 6). This is followed by a fragmentation of the cell membranes. In certain cases there is a swelling of the epidermal cells to such an extent that the cells lose their cuboidal shape, and in some instances assume an irregular "foamy" appearance (Fig. 8). The cilia are shed or broken, and the rhabdites are thrown off as the cell membranes fragment. The cells thus affected collapse readily and cytolyze. In a number of cases where the outline of the epidermal cells is lost completely, a mass of nuclei, fragments of rhabdites, and protoplasmic granules are left lying on the basement membrane. Breaks may occur in a number of regions simultaneously; in the worms sectioned they appear first more often in the dorsal posterior region. This may be due to the internal location of the proboscis at this point.

The parenchyma cells do not appear to increase in size or number as a result of the experimental treatment. The cytological changes are first apparent in the nuclei. The chromatin granules scatter, and the nuclei take a light stain. The protoplasm breaks away or cytolyzes, and the nuclei disintegrate. Although certain bodies scattered in the débris of the disintegrated gut appear to be parenchyma cells, it is possible that they are the nuclei of the broken gut cells. Both look much alike and measure approximately the same.

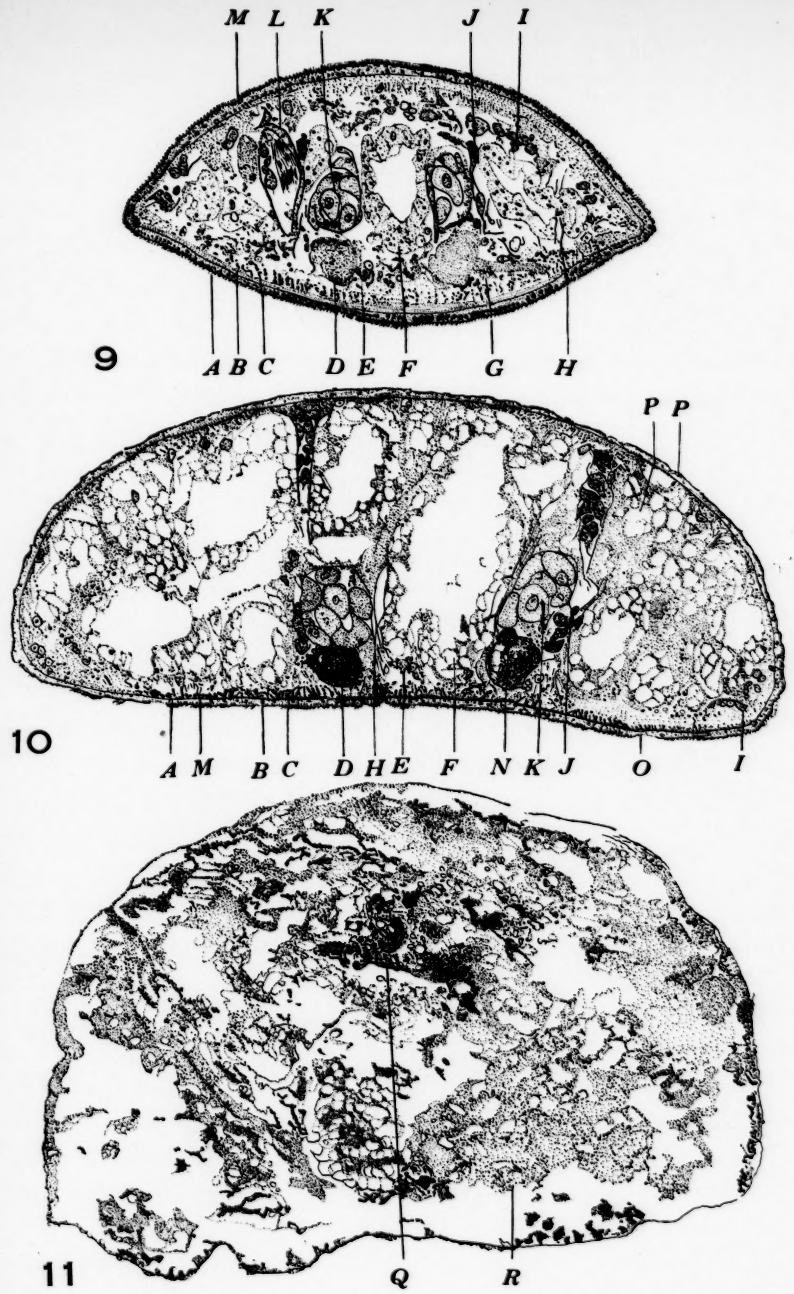
The cerebral ganglia and nerve cords are very resistant to cytolysis, but finally fragment by the formation and coalescence of vacuoles. When other tissues are badly affected, the ganglia and nerve cords show only slight changes. In certain cases where all tissues except a fragmented proboscis were completely disintegrated, a highly vacuolated brain and a portion of one nerve cord could be identified. The eyes show about the same resistance as do the nerve cords and break up by fragmentation and granulation.

The proboscis is one of the last organs to show the effect of this experimental treatment. The sheath surrounding it is delicate and breaks as the gut disintegrates. The first step in the histolysis of the

proboscis is the appearance of vacuoles in the protoplasm between the oblique strands of muscles and near the outer periphery. These vacuoles coalesce to form long irregular spaces between the muscle strands. Following these changes, the muscle strands collapse and vacuoles appear in the circular and sphincter layers of muscles. Vacuoles are seldom noted in the epidermal cells, perhaps because of the fact that the nuclei of these cells lie imbeded in the parenchyma beneath the circular muscles and are attached to the cell body by slender strands of protoplasm. The epidermis breaks and sloughs off in small patches, carrying both rhabdites and cilia. These changes in the proboscis may take place earlier if the proboscis projects through the body wall. There is slight evidence that the probosci increase in size during the disintegrative processes, though this evidence is not conclusive.

In the normal worms there are a number of marginal gland cells which, classified according to their staining reaction, consist of two eosinophilic types (red-staining), one brown-staining type, and one larger blue-staining type. The blue-staining type of gland cell which appears along the dorsal margin of the body and seems to be associated with the formation of testes or vitellaria. The eosinophilic type appear to increase in number in worms from hypotonic solutions, but no increase was noted in the other types. The blue- and brown-staining types of cells cytolyze at the same time as the muscular and reproductive tissues, while some of the red-staining cells remain until nearly all tissues are reduced to fragments. In a number of cases there are large groups of red-staining gland cells located in the dorsal region of the body which closely resemble the eosinophilic type of marginal gland cell but which prove to be immature vitellarian cells.

Finally, the whole process of disintegration in *Procerodes wheatlandi* may be summed up as a process involving vacuolation in most tissues, accompanied by fragmentation and cytolysis. Cross-sections are shown of a normal worm, a worm from distilled water, and a worm from a water extract of *Procerodes* (Figs. 9–11), to illustrate the normal appearance, the early changes caused by a hypotonic environment, and a stage of complete cytolysis. In a number of tissues, swelling seems to accompany vacuolation, as shown by micrometer



FIGS. 9–11.—Cross-sections through anterior region of worm showing gross changes. (Low power.)

FIG. 9.—Normal.

FIG. 10.—Initial degeneration; distilled water—2 hours 5 minutes.

FIG. 11.—Late stage of cytolysis; *Procerodes* extract—49 hours.

A, epidermis; *B*, circular muscle; *C*, longitudinal muscle; *D*, ventral nerve cord; *E*, parenchyma cells; *F*, branch of gut; *G*, branch of ventral nerve cord; *H*, dorso-ventral muscle strands (oblique); *I*, marginal glands; *J*, vitellaria; *K*, ovary; *L*, testis; *M*, rhabdites; *N*, oviduct; *O*, fragmented epidermis; *P*, vacuoles; *R*, cytolized cerebral ganglion; *S*, food granules.

measurements. However, owing to the fact that the worms may have contracted during the fixation process, these indications cannot be taken as conclusive evidence; there is no doubt but that the whole worm becomes greatly swollen.

From the tabulated data the following sequence of histolysis is established: gut, muscle, reproductive organs and associated ducts and glands, epidermis, parenchyma, ventral nerve cords, proboscis, brain, and eosinophilic marginal gland cells. It must be remembered that the whole process of histolysis is taking place simultaneously, and that the majority of the tissues are affected before any one is completely disintegrated. The non-cellular basement membrane survives all cellular tissues, except in the case of large wounds, and serves to hold the general shape of the body when the cellular tissues have largely disintegrated.

B. HISTOLOGICAL CHANGES IN WORMS FROM HYPOTONIC SEA WATER VERSUS WORMS FROM HYPOTONIC CONDITIONED WATER

Six detailed comparisons were made of pairs of worms, using a hypotonic sea-water control contrasted with a worm from a similarly hypotonic, biologically conditioned medium. Each contrast was made between worms where the time of exposure and the electrolytic concentration of the medium were as nearly equal as possible. The experimental time varied from 4.25 hours to 49 hours, and the electrolytic concentration of the media varied from that producing a resistance from 1,700 ohms to 3,500 ohms. The different media used for tests against the controls were *Euplanaria novangliae* (= *P. maculata*) culture medium and *Procerodes* extract. One of these typical comparisons is shown in Table I.

The results given in full in Table I are summarized in Table II together with three other such detailed comparisons in which the worms of any given pair were sectioned after having been exposed for approximately the same length of time on the same day to a hypotonic sea-water control, or to a biologically conditioned medium of the same initial concentration of electrolytes. In each case the histological analysis confirms the hand-lens survey which preceded sectioning, although, as might be expected, small epidermal lesions are apparent in the microscopic examination of the sections which were

TABLE I

A DETAILED HISTOLOGICAL COMPARISON OF *Procerodes* EXPOSED TO HYPOTONIC SEA-WATER AND TO FRESH-WATER PLANARIAN CULTURE, BOTH WITH AN INITIAL RESISTANCE OF 2,700 OHMS

The worms were killed after an exposure of 4.25 hours.

VII. Hypotonic sea-water control	VIII. Planarian culture medium
ANTERIOR REGION	
EPIDERMIS	
Broken in "patches." Badly broken in region above the proboscis. Cells vacuolated.	Broken laterally in the proboscis region. Cells vacuolated. "Foamy" in lateral regions.
RHABDITES	
Many present dorsally. Fewer in ventral region.	Few present and seem "fragmented" dorsally.
CILIA	
Present dorsally and on lateral-ventral regions.	Present in ventral region.
MUSCLES	
Swollen and vacuolated.	More enlarged and vacuolated than in VII.
CEREBRAL GANGLION	
Normal appearance.	Normal appearance.
GUT	
Full of vacuoles. Pulled loose from attachment in some regions.	Comparable to VII in this region.
OVARY	
Ovary not broken. Eggs affected in the nuclear region.	Ovary broken out of place. Eggs affected in the nuclear region.
MID- AND POSTERIOR REGIONS	
EPIDERMIS	
Badly broken from proboscis region to the posterior end, especially in dorsal region	Collapsed at the posterior end, but in better condition than VII.
MUSCLE	
Badly broken and fragmented in the posterior regions.	Enlarged and vacuolated as above.
NERVE CORDS	
Badly vacuolated posteriorly.	Only slightly vacuolated.

TABLE I—*Continued*

VII. Hypotonic sea-water control	VIII Planarian culture medium
PROBOSCIS	
Vacuolated badly in some regions. Distorted.	Slightly vacuolated and wrinkled. Torn loose at root.
VITELLARIA	
Granules clumped, giving vacuolated appearance. Cells scattered.	Granules clumped. Cells not broken apart; few in number.
TESTES AND SPERM	
All broken up in the posterior region. Sperm affected.	Testes not broken. Sperm slightly twisted.
ACCESSORY REPRODUCTIVE ORGANS	
Vas deferentia broken. Sperm granulated. Penis, glandular organ, and all ducts disintegrated.	Vas deferentia and sperm in better condition than in VII. Penis and glandular organ broken though distinguishable. All ducts distinct.

SUMMARY.—The structures in the anterior end are affected in about the same degree, except that the epidermis of VIII has fewer breaks. VIII is in better condition in the mid- and posterior regions, as is shown by the condition of the epidermis and reproductive organs.

not visible at the time of the examination of the living material with the lower magnification. The histological picture was, however, not always so distinct as might have been expected from the difference in the physiological condition of the worms. This was especially noticeable between worms XXIII and XXIV.

In each case the worm from the biologically conditioned medium was in better histological condition than was the comparable control worm, and in each case more of the worms were living in the conditioned medium at the time the worms were fixed for sectioning; and the whole lot survived longer, on the average, than did those in the accompanying controls.

The chief differences in the stages of cytolysis are expressed in the epidermis, nerve cords, reproductive organs, muscle, and gut. The epidermis of worms from biologically conditioned media is less vacuolated and fragmented, and shows fewer large breaks than does that of the controls with comparable exposure. The reproductive organs of worms from biologically conditioned media may also show an ad-

vantage over those of the controls. The ovaries do not show a decided difference unless a large break occurs in the epidermis dorsal to them. The testes, vasa deferentia, and all accessory organs and ducts show a marked difference in the stage of cytolysis, especially

TABLE II

A SUMMARY OF THE HISTOLOGICAL COMPARISONS OF *Procerodes* EXPOSED SIMILARLY TO BIOLOGICALLY CONDITIONED MEDIA AND TO COMPARABLE CONTROLS

Sectioned Worms	Initial Resistance of Medium (Ohms)	Worms Exposed to—	Percentage of Worms Alive	Recorded Condition When Killed	Exposure Time (Hours)	Mean Survival of Exp. Mates. (Hours)	Histological Summary
VII.....	2,700	Control	30	Cytolyzed	4.25	3.73	Poorer condition than VIII
VIII.....	2,700	*Euplanaria novangliae* culture medium	80	Intact	4.25	4.60	Better condition than VII
XIV.....	1,700	Control	0	Cytolysis started	8.25	4.26	Poorer condition than XV
XV.....	1,700	*Procerodes* extract	25	Intact and moving	8.25	7.13	Better condition than XIV
XVI.....	2,500	E. *novangliae* culture medium	15	Intact and moving	8.25	5.15	Better condition than XVII
XVII....	2,500	Control	0	Cytolysis started	8.25	3.71	Poorer condition than XVI
XXIII...	2,700	Control	25	Cytolysis started	7.67	6.63	Poorer condition than XXIV
XXIV...	2,700	*Procerodes* extract	90	Intact and moving	8.33	62.81	Better condition than XXIII

in worms in which the epidermis has broken greatly or collapsed. Large epidermal lesions occur more often in the control worms, thus giving the worms from biologically conditioned media an advantage. The muscle layers of control worms are usually more vacuolated and broken than those of worms from conditioned media. The gut also shows a difference in a number of cases, though it is not as consistent

an indicator as the foregoing tissues. In each group where pairs of best examples of sectioned worms from each medium were contrasted after comparable exposures, in five cases out of six those from the biologically conditioned media showed definite cytological advantages over the controls.

DISCUSSION

The histological changes in *Procerodes wheatlandi* produced by exposure to the extremely hypotonic media used consist largely of vacuolation followed by fragmentation and cytolysis. The discovery of the phenomenon of vacuole formation in cells exposed to hypotonic media is not of recent date; it appears from the summary given by Heilbrunn (1928, p. 234) to have been well known to botanists in 1867.

The early vacuolation of gut cells of *Procerodes wheatlandi* is in accord with the situation in the related *P. ulvae* (=*Gunda ulvae*), as shown by Lloyd (1914), Weil and Pantin (1931), and Beadle (1931, 1934). In brief, Beadle (1934) reports that when *P. ulvae* is transferred to about 10 per cent sea water there is an initial inflow of water through the epidermis into the parenchyma and that swelling results. This water is then taken up by the gut cells and is collected in the intracellular vacuoles. After the original inflow of water, the epidermis becomes less permeable. In calcium deficiency, the epidermis loses its impermeability; and with lowering of the osmotic pressure below that of 2 per cent sea water, swelling and final disintegration takes place in all the body cells, including those of the epidermis and the proboscis.

Hansborough (1931) found that upon exposure of *Procerodes wheatlandi* to extremely hypotonic media not only the gut cells, but also the epidermal cells, became enlarged and vacuolated, and certain other cells showed changes in size and character. In the present study, which includes worms from 1.16 hours' exposure to 49 hours exposure to hypotonic media, it has been noted that nearly all tissues become vacuolated before they entirely disintegrate. The fragmentation of tissues follows the vacuolation process as a matter of course when the worms are exposed to hypotonic media for a long period.

There is also some evidence that certain tissues other than the gut swell during the disintegrative process. Micrometer measurements

of epidermal cells, eosinophilic gland cells, and the proboscis show that in certain cases these structures are swollen. In the epidermis measurements, fifteen cases showed a mean increase of 28.23 per cent, while ten cases showed no increase or a mean decrease of 19.70 per cent. The increase in width seems to be the result of the formation of large vacuoles in the epidermal cells. Measurements of the proboscis showed a mean increase in width in twenty cases of 12.75 per cent, and in six cases a mean decrease in width of 11.80 per cent. Increase in the size of gut cells is shown in camera-lucida drawings made from approximately the same level of the body and at the same magnification. The increase and decrease in size of the structures could not be definitely correlated with exposure to any particular type of medium.

All rhabdites are not completely shed from the epidermal cells until a late stage of cytolysis. In worms in distilled water, the rhabdites are nearly all shed by 1.16 hours; those in hypotonic sea water, by 27.5 hours; and those in hypotonic biologically conditioned water, by 44 hours. The rhabdite-producing gland cells disintegrate at approximately the same time as the muscle, so that some of the epidermal cells may be replenished with rhabdites before the producing cells cytolyze. However, there is no definite proof that this does occur. The mucus produced by the dissolving of the rhabdites in water is commonly thought to be protective. However, mucus produced by dissolving desiccated parotid gland in distilled water does not prove to be protective from hypotonic water (Allee, 1933), and recent work by Oesting and Allee (1934) indicates that the protective value of mucus from hypotonic water is largely—perhaps wholly—associated with the calcium content.

The sequence of tissue disintegration depends in part on the compactness of construction of the various tissues, and in part probably on differences in physiological activity. The gut, composed of loosely arranged tissues, is the first to begin and the first to complete cytolysis; the proboscis withstands severe treatment longer than the other organs. The nerve cord and epidermis appear to be more compact than are the longitudinal muscles. The epidermis usually has a few breaks before the body muscle vacuolates greatly, but the latter appears to precede the epidermis in final disintegration.

The epidermis seems to control the intake of water, and thus controls one of the factors of disintegration. The internal tissues cytolyze much more rapidly if large breaks occur in the epidermis. This is clearly shown by the rapid cytolysis of the tissues directly beneath the breaks. Since the proboscis is well innervated and both the nervous system and the proboscis resist cytolysis, it seems probable that movements of the proboscis hasten the collapse of the tissue above it. This accounts for the numerous occurrence of large breaks in the epidermis in the dorsal posterior regions of the body.

It happens that worms which were sectioned and carefully studied showed more epidermal lesions in the posterior dorsal region. This, however, does not coincide with the much more extensive experience of Allee on this point which has not been published hitherto. Allee made his examinations with a Hastings triplet lens magnifying seven times, and recorded the initial location of the first visible lesion for 1341 *Procerodes* exposed to extremely hypotonic sea water, a part of which had been biologically conditioned. These first evidences of surface cytolysis all appeared on the dorsal or lateral surfaces. In part, the fact that initial lesions are not recorded from the ventral surface may be due to the fact that the worms are sometimes still attached by the ventral surface; more frequently they are lying on their sides, so that ventral lesions could easily be detected. In none of these were indications of ventral epidermal breaks found until relatively late in the disintegrative process. This is evidence of a greater toughness of the ventral epidermis, such as would be anticipated on general grounds.

Of the recorded lesions, 36.3 per cent occurred in the anterior third of the worm, usually at the very anterior tip or at the tips of the tentacles or in the dorsal epidermis near the eyes; 24.8 per cent were found in the middle third; 23.9 per cent, in the posterior third; and 5.0 per cent revealed initial cytolysis simultaneously in the anterior and the posterior regions. Similarly, Oesting and Allee in 1934 found that, of 586 worms for which records were kept, 47.2 per cent had their initial lesions in the anterior end, 23.3 per cent in the mid region, and 27.3 per cent in the posterior part. The remaining 2.2 per cent showed two initial lesions in some one of the possible combinations of these regions.

The preponderance of first external evidence of cytolysis in the anterior end is the more significant since the posterior end is somewhat differentiated as an organ of adhesion and is muscularly an active region, and further, since the presence of the large muscular pharynx is a potent source of epidermal lesions in the more posterior region of the worm. Neither the observations of Allee nor the histological studies here reported give a basis for an explanation of the greater initial susceptibility of the epidermis of the head region. The observed facts suggested the existence of some sort of an axial gradient such as has been demonstrated frequently for diverse organisms by Child, Hyman, and others.

The parenchyma cells do not seem to act as phagocytic agents during histolysis, as they do during the histological changes previous to starvation (Lloyd, 1914). They do not appear to migrate into the disintegrating tissues; nor do they show a pronounced increase in number.

The histological evidence of a definite protection afforded to the worms in the biologically conditioned hypotonic sea water adds support to the findings of Allee (1933), as judged by the length of life of worms in various solutions. The worms in hypotonic biologically conditioned media exhibit a better state of histological preservation than do the comparable controls. The epidermis of the controls in approximately all cases showed more fragmentation and large breaks than did the worms in conditioned solutions. The gut, muscle, and reproductive structures also showed more advanced stages of cytolysis in the control worms. Allee (1933) showed that the protection of the worms in the hypotonic biologically conditioned media is not due entirely to simple differences in osmotic pressure, and that the protection is related to some physiological effect produced by the conditioning of the medium. More recently, Oesting and Allee (1934) have demonstrated that the protection furnished by biological conditioning can be explained on the basis of the increased calcium content of the conditioned water. The present study does not attempt to present a further explanation of the protective phenomena; it is concerned only with the histological picture of internal changes in *Procerodes wheatlandi* exposed to these and other similarly hypotonic solutions.

Underlying the work here summarized, there is the conviction that, whenever possible, physiological studies such as those of Allee on *Procerodes* should be supplemented and verified by histological examinations. These not only serve to test the accuracy of the more superficial observations possible on fresh material but prepare the way for a more accurate picture of the physiological processes involved. Without such studies we should not have suspected the early vacuolization of the cells of the gut or their probable rôle in protection from brief exposure to hypotonic media. The present observations, in these and in other points in so far as they deal with the same material, support the work of Beadle (1934), who used similar methods in his studies on the related worm, *Procerodes ulvae*.

SUMMARY

1. Certain histological changes that occur in the tissues of *Procerodes wheatlandi* upon exposure to hypotonic media are described, and the sequence in cytolysis of their tissues is established.

2. There is no qualitative difference between worms exposed to biologically conditioned media, as compared with those equally exposed to similarly extremely hypotonic media which were not biologically conditioned.

3. A comparison of the relative state of preservation of the tissues of the worms from the two types of treatment does reveal quantitative differences in that, with comparable exposure and other conditions being equal, worms from biologically conditioned media are in a better state of histological preservation.

4. This histological study confirms the conclusions which Allee drew from his more superficial examinations. The worms for this study were taken from his experiments.

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THE ACTION OF SODIUM, POTASSIUM, CALCIUM, AND MAGNESIUM IONS ON THE PLASMAGEL OF AMOEBA PROTEUS AT DIFFERENT TEMPERATURES[1]

(Two figures)

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THE colloids of protoplasm are undoubtedly sensitive to alterations in the environment. Changes in protoplasmic viscosity are readily induced by a variety of chemical reagents or by such a variable physical factor of the environment as temperature. A monograph by Heilbrunn (1928) gives a review of the effect of temperature and of various cations on the viscosity of different types of protoplasm. More recently, Costello (1934) has plotted a viscosity-temperature curve for *Arbacia* eggs, while Heilbrunn and Daugherty (1931, 1932) have studied the effect of the chlorides of Na, K, Mg, and Ca on the protoplasm of *Amoeba dubia* and *A. proteus*. It is the purpose of these experiments to determine to what extent the viscosity-temperature relationship of the plasmagel is altered by immersing amoebae in solutions of the above-mentioned salts. This work was undertaken at the suggestion of Dr. L. V. Heilbrunn.

MATERIAL AND METHODS

The material used in the earlier stages of this experiment was a strain of *Amoeba proteus* Pallas obtained originally from Dr. J. A. Dawson. Another strain of *A. proteus* (*Chaos diffluens* Sch.) was later obtained from Dr. A. A. Schaeffer. Subcultures were subsequently prepared by transferring from 50 to 100 amoebae from the original culture to a flat-bottomed dish containing 50 cc. of water redistilled over quartz. Prior to inoculation with amoebae each culture was provided with a supply of bacteria and *Chilomonas paramecium* taken from the original culture. A short piece of hay and a

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grain of wheat, both sterilized by boiling, completed the culture medium. Additional water, in small quantities, was added from time to time in order to compensate for loss by evaporation. Cultures were kept in diffuse daylight at a temperature of 18°–22° C. Within 3–4 weeks such cultures usually contained a sufficient number of amoebae for experimental purposes, and in some cases provided amoebae daily for a month. The pH of the cultures varied from 6.8 to 7.2.

Determinations of the "centrifuge value" of the plasmagel of *Amoeba proteus* were made by means of the centrifuge method (Heilbrunn, 1928). The use of the term "centrifuge value," which may or may not be a true measure of viscosity, has been adequately discussed by Heilbrunn and Daugherty (1932), who define it as the number of seconds of centrifuging necessary in order to shift all of the crystals of the plasmagel into one-half of the cell. The use of the term "viscosity" throughout this paper denotes the centrifuge value as defined above. The centrifuge value varies between different cultures and even with the same culture at different times. In order to secure comparable results, it is thus necessary to select arbitrarily a "normal" centrifuge value which can serve as a standard of comparison for future measurements. In the determination of the normal centrifuge value it is not essential that all of the amoebae in any one culture should have the same value, because a certain amount of variability is to be expected in all living material. For the purpose of this experiment a culture at a temperature of 20.5° C. was considered normal when the crystals were displaced halfway in at least 80 per cent of the amoebae after 100 seconds of centrifuging at the rate of one turn of the handle in 2 seconds. The Emerson hand centrifuge used in these experiments developed a force 2,474 times gravity when the handle was turned at the rate of one turn every 2 seconds. Cultures in which more than 20 per cent of the amoebae showed a variation of more than 10 turns on either side of the normal were not considered suitable for experimentation. Table I is a representative data sheet which illustrates the manner of arriving at a given value.

From Table I it is quite evident that 95 seconds of centrifuging at the rate of one turn every 2 seconds is sufficient to cause the crystals of 80 per cent of the amoebae to be displaced halfway. Less than 95

seconds of centrifuging will not produce the desired result in 80 per cent of the amoebae; on the contrary, more than 95 seconds of centrifuging not only increases the percentage ratio considerably beyond the required 80 per cent, but in many of the amoebae the crystals are so tightly packed that they occupy less than one-half of the cell volume. The location of each point on the viscosity temperature curves was determined in essentially the same manner; each measurement is thus the result of a series of observations on small lots of amoebae, all of which have been subjected to a similar set of experi-

TABLE I

TESTS OF CENTRIFUGE VALUE AT 20.0° C. CULTURE XX, MARCH 23, 1934; VISCOSITY, 95 AT 20.0° C.

Immersion Time (Minutes)	Number Observed	Amoebae with Crystals Displaced Halfway	Centrifuging Time (Seconds)
90.....	8	4	80
90.....	15	11	90
90.....	17	13	90
90.....	12	9	90
90.....	14	11	90
90.....	13	12	100
90.....	8	7	95
90.....	18	16	95
90.....	22	19	95

mental conditions. Ordinarily, a total of 50–250 amoebae was used during the determination of a given centrifuge value.

The amoebae were brought to the desired temperature by transferring them from the culture to capillary tubes, which were then partially immersed in water contained in larger tubes surrounded by water of the thermostat at the desired experimental temperature. By placing the capillary tubes in the bath at regular intervals, tests could be made on a series of amoebae all of which were immersed for the same length of time. At the end of the period of immersion (90–95 minutes) the capillary tubes were removed, dried by wiping with a cloth, and immediately centrifuged at the same temperature. Room temperature was maintained within 1° of the experimental

temperature either by allowing cool air from the outside to enter the room by way of an open window or by heating the room with steam heat. During the early stages of the experiment the necessity of maintaining the room temperature at approximately the temperature of the experiment was not clearly recognized; consequently, a number of observations were made which later had to be discarded when it was found that they did not represent the true centrifuge value obtained by centrifuging the amoebae at the experimental temperature.

Occasionally cultures of amoebae were found to be unsuitable for experimental purposes because of the presence of a yellow substance which impeded the normal displacement of granules under the influence of centrifugal force. The nature of this substance is unknown except that centrifugal force causes it to accumulate in that part of the amoeba which is free from granules.

The centrifuge values of amoebae immersed in the various salt solutions were determined in the same manner as the centrifuge values of amoebae immersed in culture fluid, with the exception that prior to immersion in the water bath the amoebae were washed twice in a solution of the salt of which the effect was to be determined. The amoebae with some of the salt solution in which they had been washed were subsequently transferred to centrifuge tubes which were partially immersed in the water bath, as stated earlier. The solutions used were M/40 NaCl (Kahlbaum) with a pH of 6.4–6.6; M/40 KCl (Kahlbaum) with a pH of 6.5–6.8; M/60 $MgCl_2$ (Merck) with a pH of 6.4–6.7; and M/60 $CaCl_2$ (Baker) with a pH of 6.5–6.6. All solutions were made up in water redistilled over quartz; the hydrogen-ion concentrations were determined with bromthymol blue against a set of La Motte standards.

RESULTS

The effect of temperature on the centrifuge value of the plasmagel of amoebae immersed in their own culture fluid is evident from an inspection of Figure 1. The maximum viscosity occurs at approximately 7° C. Below 7° the centrifuge value declines rapidly; above 7° there is a similar tendency for the centrifuge value to decrease, but less rapidly than at the lower temperatures. As compared with

higher temperatures, the period from 7° to 10° C. is comparatively ineffective in reducing the viscosity; however, between 10° and 20° C. the viscosity decreases progressively with temperature increments.

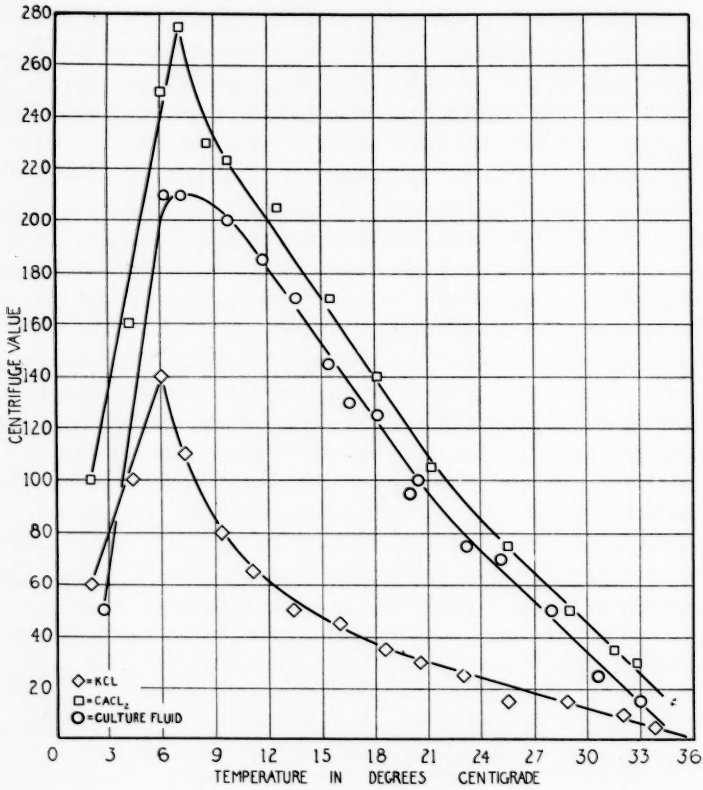

FIG. 1.—Viscosity-temperature curves of plasmagel of *Amoeba proteus*

Although the centrifuge value also shows a progressive decrease between 20° and 33° C., these changes are not of the same degree of magnitude as those shown between 10° and 20° C.

In general, these results confirm those of an earlier experiment (Thornton, 1932) in which the centrifuge value showed a progressive

decrease between 5° and 30° C. In the earlier experiments the amoebae were centrifuged at room temperatures of 18°–24° C., which would tend to alter the slope of the curve as well as to displace the point of maximum centrifuge value.

The individual effect of each of the four cations can best be understood by eliminating from consideration those obvious similarities

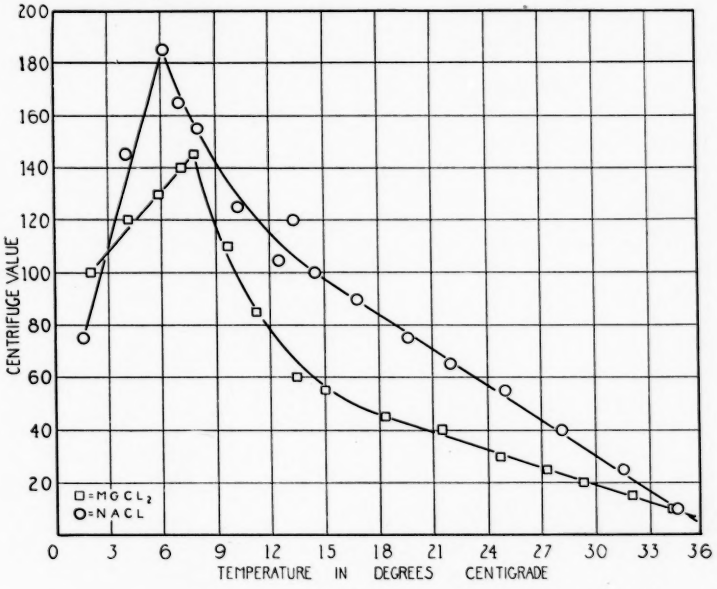

FIG. 2.—Viscosity-temperature curves of plasmagel of *Amoeba proteus*

between each of the curves which may reasonably be ascribed to the effect of temperature—the only factor common to each of the curves. In the case of each of the salt solutions and of the culture medium, which may be considered as a balanced salt solution, there is very little variation in the fundamental form of the viscosity-temperature curve (Figs. 1 and 2). Each curve shows a point of maximum viscosity at 6°–8° C.; a decrease in viscosity may be induced either by lowering or raising the temperature beyond this point; and finally, for at least the upper half of the biokinetic range, temperature incre-

ments result in progressive decreases in the centrifuge value. However, while the general form of each of the curves is similar, they each exhibit important variations in magnitude which are quite distinctive for the individual salt under consideration. Hence, if amoebae in culture solution are representative of the normal viscosity-temperature relationship, a comparative inspection of the five curves would indicate that the primary effect of Ca is to increase the viscosity of the plasmagel; whereas K, Mg, and Na act to decrease the normal viscosity. Amoebae immersed in solutions of KCl exhibit a greater decrease in viscosity than those which are exposed to a solution of $MgCl_2$, while NaCl is even less effective than $MgCl_2$. It is interesting to note that the three cations which induce a decrease in the centrifuge value are also effective agents in retarding the rate of amoeboid movement. The potassium ion, which causes the greatest drop in the centrifuge value, is likewise the most effective agent in retarding amoeboid movement. After 30–45 minutes immersion in M/40 KCl at room temperatures, the amoebae withdraw their pseudopods and there is a complete cessation of amoeboid movement. Solutions of M/60 $MgCl_2$ are less effective than KCl in preventing amoeboid movement; while in solutions of M/40 NaCl, the amoebae continue to move almost normally. While observations at the lower temperatures were not extensive, they indicate that amoeboid movement is completely inhibited at temperatures near 0° C. The temperature at which movement first becomes visible was not determined. In all cases below 30° C. the amoebae recovered when returned to the culture fluid. However, the resistance to heat was considerably lowered when the amoebae were immersed in KCl at temperatures above 30° C. In such cases first the amoebae swelled considerably; then the granules dissolved in the cytoplasm and the amoebae assumed a dirty yellowish tinge. The membranes of such specimens frequently ruptured, permitting the contents to escape.

DISCUSSION

Protoplasmic colloids respond to increases in temperature either by showing a progressive decrease in viscosity or by passing through a point of maximum viscosity. The experimental evidence for both

types of response has been summarized by Heilbrunn (1928). In an earlier paper Thornton (1932) arrived at the conclusion that at temperatures above 5° C. the viscosity of the plasmagel of *Amoeba proteus* decreased progressively as the temperature increased. The significance of a slight drop in viscosity at temperatures below 5° C. was not recognized at that time because of the error involved in making determinations of the centrifuge value at room temperature. The results of the present series of experiments indicate quite clearly that low temperatures exert a liquefying effect on the plasmagel of *A. proteus;* additional support is thus given to the view that some types of protoplasm may pass through a point of maximum viscosity as the temperature increases progressively.

The effect of salt solutions over the entire biokinetic range has not previously been studied. The present experiments indicate that immersion of amoebae in salt solutions does not appreciably alter the fundamental effect of temperature in determining the form of the viscosity curve. On the contrary, the individual effect of each of the salt solutions appears to be superimposed on the temperature effect, except possibly at temperatures below 6° C., where the relationship does not quite hold. The ability of K, Mg, and Na to decrease the viscosity and of Ca to increase the viscosity of the plasmagel has been shown previously by Heilbrunn and Daugherty (1932), who also found (1931) that the two monovalent cations produced an increase in the viscosity of the plasmasol of *Amoeba dubia*, but that the two divalent cations produced a decrease.

Inasmuch as the rate of amoeboid movement at various temperatures is probably the resultant of the interaction of a number of variable factors, some of which have not yet been thoroughly studied, it would be interesting to know the effect of each of the foregoing cations on the protoplasm of *Amoeba dubia* at different temperatures.

SUMMARY

A series of curves has been presented in which the relation of temperature and various salts to the centrifuge value of the plasmagel of *Amoeba proteus* has been shown as measured by the centrifuge method.

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